THE UNIVERSITY *of* EDINBURGH

African trypanosomes; a model for the improvement of molecular diagnosis of blood borne parasites

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Declaration

I declare that the research described within this thesis is my own work and that this thesis is my own composition

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Contents

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Abstract

Historically, diagnosis has relied on clinical signs of disease, microscopy and serological testing. However, these approaches have a number of drawbacks for example, differential diagnosis, low sensitivity (microscopy) and the inability to differentiate past from current infections (serology). In the past decade the use of molecular techniques, such as the polymerase chain reactions (PCR) have gained favour. Many research groups have used these techniques to study the molecular epidemiology of diseases in sub-Saharan Africa. Such methodologies rely on the detection of genetic materials and as such are reliant on the specificity of their components and the quality of the starting materials. It is the aim of this thesis is to demonstrate improvements that can be made to sample collection that will help to enhance the reliability of these tests and highlight the importance of the diagnostic parameters. The model that I will use to demonstrate these improvements are African trypanosomes, these are the causative agents of sleeping sickness in humans and nagana in animals, and are wide spread across much of sub-Saharan Africa. My work will be presented as three main sections:

Firstly, a comparison of the suitability of various different approaches to cattle blood sample collection – including the genetic materials prepared directly in the field and the use of Whatman FTA®cards – in terms of the provision of appropriate materials for molecular screening will be presented. It was found that uneven distribution of genetic materials occurs across the surface of the FTA®cards due to the matrix chemistry. Therefore suggestions for improvements for the preparation of materials to be stored on these cards and their downstream application are made.

Secondly, a comparison between the specificity of the pan-*Trypanosoma* ITS-PCR reaction and the species-specific reactions is made. The ITS-PCR has gained favour in recent years as it is reported to be capable of identifying a wide range of trypanosomes, as this is a single nested PCR reaction the reduction in time and cost has been very appealing to researchers in this field. My work suggests that this test is not reliable in terms of the accurate detection of trypanosomes species, and in fact on a direct comparison of 969 samples, 37 parasitic events where identified by this approach compared to 197 when species-specific tests were applied.

Thirdly, based on my findings from the previous two chapters I present two case studies, the first of which looks to evaluate the impact on the prevalence of trypanosome species in cattle after drug treatment during the Ugandan, Stamp Out Sleeping sickness (www.sleepingsickness.com) campaign. The results of this case study highlight the importance of understanding the relationship that occurs between trypanosome species in mixed infections, my second case study therefore looks to quantifying the infection load of *Trypanosoma brucei* and *T. congolense* within the midgut of their insect vector (*Glossina morsitans morsitans*) using qPCR.

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Abbreviations used

Ab	Antibody
Ag	Antigen
BCT	Buffy coat technique
Bst	*Bacillus stearothermophilus*
bp	Base pair
CATT	Card agglutination trypanosomiasis test
CFT	Complement fixation test
CNS	Central nervous system
CSF	Cerebro-spinal fluid
DDT	Dichlorodiphenylethane trichloroethylene
DI	Diminazine-aceturate
DNA	Deoxyribo-nucleic acid
ECF	East coast fever
ELISA	Enzyme linked immunosorbent assay
ES	Expression sites
ESAG	Expression site associated genes
FFLB	Fluorescent fragment length bar-coding
FITCA	Farming in tsetse controlled areas
fg	Femtogram
FRET	Fluorescence resonance energy transfer

FTA	Flinders technology Australia
GPI	Glycosyl-phosphatidyl-Inositol
GSH	Glutathione
HAT	Human African trypanosomiasis
HCl	Hydrochloride
HCT	Haematocrit centrifugation technique
HIV	Human immunodeficiency virus
HRT-1,2,3,	Histidine rich protein
HVR	Hypervariable region
IFAT	Indirect fluorescent anyibody test
I/M	Intra-muscular
IPTG	Isopropyl β-D-thiogalactopyranoside
ISM	Isometamedium chloride
ITS	Internal transcriped spacer
I/V	Intra-venous
kDNA	Kinetoplast deoxyribo-nucleic acid
KIVI	Kit for *in vitro* isolation of trypanosomes
LAMP	Loop mediated isothermal amplification
LB	Luria Bertani
LED	Light emitting diode
NASBA	Nucleic acid sequence based amplification
NCBI	National Centre of Biotechnology Information
ng	Nanogram

NPV	Negative predictive value
NTS	Non transcriped spacer
PBS	Phosphate buffer saline
PCV	Packed red cell volume
PCR	Polymerase chain reaction
pg	Picogram
pLDH	Parasitic specific lactate dehydrogenase enzyme
PLC	Phospholipase C
PM	Peritrophic membrane
PPV	Positive predictive value
QPCR	Quantitative polymerase chain reaction
QT-NASBA	Quantitative nucleic acid sequence based amplification
RBC	Red blood cells
RDT	Rapid diagnostic tests
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribo-nucleic acid
S/C	Sub-cutaneous
SDA	Strand displacement amplification
SDS	Sodium dodecyl sulfate
SIT	Sterile insect technique
SOS	Stamp out sleeping sickness
3SR	Self sustained sequence replication
SRA	Serum resistance associated gene

Ssu-rRNA	Small sub-unit ribosomal ribo-nucleic acid
Taq	*Thermus aquaticus*
TgsGP	*Trypanosoma brucei gambiense* specific glycoprotein
UV	Ultra-violet
VSGs	Variable surface glycoproteins
WBC	White blood cells
WHO	World health organization
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyransoide

1 Chapter one

Development of diagnostic strategies for blood borne parasitic diseases

1.1 Introduction

Diagnosis is an essential element in the management of disease, both at the level of individual patient care and at the level of disease-control in populations. Diagnostic tests should be simple, rapid, specific and highly sensitive. They also should ideally differentiate between closely related parasites, especially if the disease syndromes they cause require different management approaches. They should be suitable for field application and the cost for these tests should be within the means of the communities affected by the disease (Nantulya, 1991).

The aetiology of infectious diseases has traditionally been investigated through the establishment of Koch's postulates (Poxton, 2005). The postulates state that an organism is causal if:

1- It is present in all cases of the disease

2- It does not occur in another disease as a fortuitous and non-pathogenic parasite

3- It is isolated in pure culture from an animal, is repeatedly passaged and induces the same disease in other animals (Koch, 1882).

Although these postulates were the basics to understand the infectivity of the causative organism, they have limitations in dealing with asymptomatic carriers. Asymptomatic carriers are those carrying the infectious agent without having clinical signs.

The diagnosis of the asymptomatic carrier is crucial because they act as a reservoir of the disease, leading to transmission and spread of the infectious agent amongst population (Falkow, 2004). Moreover, the transition from asymptomatic carrier to clinically sick is related to failure in the host immune response rather than an increase in pathogenicity of the infectious agent (Evans, 1991; Poxton, 2005).

Blood borne parasites were traditionally diagnosed using microscopy to directly observe the causative agent within blood or through the use of serological approaches for the detection of either antibodies to or antigens from the pathogen in the host serum. However, recently the explosion of new techniques, due to rapid advances in molecular biology, has provided a battery of novel approaches and technologies, which can be applied to more practical issues such as diagnosis and research studies (Hide and Tait, 1991; Nantulya, 1991).

The use of molecular approaches such as polymerase chain reaction (PCR) is important for the detection of low levels of infectious agents circulating in the blood and more importantly for the identification of asymptomatic carriers. An example of this would be the identification of animals carrying human infective *Trypanosoma brucei rhodesiense* which is non pathogenic for the animal host, but fatal to human (Eisler *et al.*, 2004).

This chapter summarises the development of diagnostic tools for the detection and characterisation of the infectious agent. The second part of this chapter describes the development of diagnostic strategies for three blood borne parasites (*Plasmodium, Leishmania* and *Trypanosoma cruzi*) from the traditional approaches of microscopy and serology to the most recent molecular tools used for diagnosis and identification of such agents.

1.2 Microscopical diagnosis

The main approach for diagnosing blood borne parasites is the examination of blood samples by microscopy. The definition of particular species within a genus is dependant on morphological criteria (Prichard and Tait, 2001). Microscopy can be performed quickly, but accuracy depends on the experience of the microscopist and quality of equipment.

Different blood films have been used for this purpose, including wet blood films and thick or thin fixed blood films stained with *Giemsa* stain. Using the wet blood film approach, a drop of blood can be examined directly using a microscope. Thick and thin blood smears fixed with methanol or acetone and stained with *Giemsa* are examined in the laboratory to detect blood parasites and determine the species involved in the infection (Hoare, 1972). Phase contrast microscopy is used to examine the details of the cells which are undetectable using bright field microscopy. The inability of the light microscope to examine the details of cells is due to the lack of contrast between structures with similar transparency and insufficiency of natural pigmentation.

Although widely used, these methods are considered to have low sensitivity and are also time consuming; therefore, these drawbacks were the main reason for research to be directed toward the detection of antibodies against the infectious agents for the diagnosis of infections.

1.3 Serology

Serodiagnosis of diseases depends mainly on antigen-antibody reaction in the blood serum. The essential element in immunodiagnosis is the availability of species-specific test antigens and/or highly specific antibodies. Because parasites contain thousands of potentially antigenic polypeptides, glycoproteins and glycolipids, many of which are shared with unrelated species and even with bacteria, there has been great difficulty in developing sufficiently specific immunodiagnostic tests (Nantulya, 1991). The advantage of using a robust serological test is that they can be used for mass screening without the need for expensive analytical equipments. However, the main drawback of serological tests is the inability of these tests to differentiate current from previous infection since antibodies frequently persist far longer than the infectious agent within the host.

1.3.1 Complement fixation test (CFT)

Complement fixation test is one of the widely used immunodiagnostic tests for the detection of antibodies or antigens for a variety of blood borne parasites. Complement is a biological substance present in the sera of normal humans and animals helping in the clearance of invading pathogens (Male *et al.*, 2006). CFT uses sheep red blood cells (RBC) as an indicator system, meaning that in a positive test, the complement is bound to the antigen-antibody complex and is not free to interact with sheep RBC which in turn settle to the bottom of the well forming a button shape. Alternatively, in a negative reaction, the complement remains free due to the absence of antigen-antibody reaction and react with the sheep RBC leading to lyses and the serum colour turns pink (Male *et al.*, 2006), as shown in Figure 1.1. This test is considered the official diagnostic test for infectious diseases such as babesiosis, brucellosis and piroplasmosis due to ease of application and suitability for in field screening (Bashiruddin *et al.*, 1999; Herr *et al.*, 1985; Luckins, 1992). However, the main drawback of this test is the lack of sensitivity especially during the late stages of disease and the difficulty encountered during the preparation of reagents.

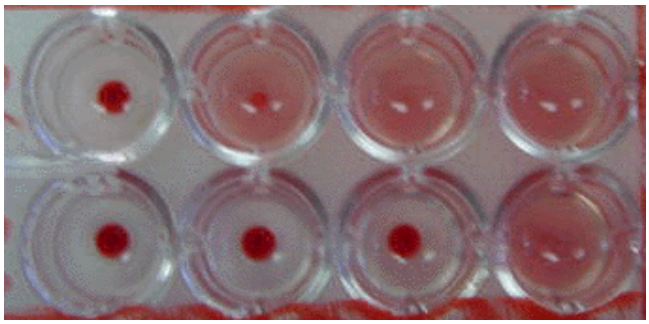


Figure 1.1: Complement fixation test results in serum sample (positive results: button shape RBC, negative results: pink color of serum)

(virology-online.com/general/Test3.htm)

1.3.2 Agglutination tests

In agglutination tests, the reaction of antibody with the antigen can be detected by agglutination or clumping of the antigen. Specific infectious agent antigen applied to the card can be used for the identification of antibodies produced against such infectious agent by observing the agglutination produced from the antigen-antibody reaction (Male *et al.*, 2006). For instance, antibodies against *Neospora caninum* (causes hind and forelimb paresis) and *Sarcocystis neurona* (causes myelitis and encephalitis), which are both protozoa, were determined in serum samples of 501 domestic cats from Brazil using the agglutination test (Dubey *et al.*, 2002). The test detected *Neospora caninum* in 11.9% of the tested sera, whereas, *Sarcocystis neurona* could not be identified.

1.3.3 Indirect fluorescent antibody test (IFAT)

In this test, blood films from infected laboratory animals are fixed and used as a source of infectious agent antigens to which antibodies in test sera may bind specifically. Bound antibodies are visualised using anti-host species immunoglobulin conjugated to a fluorescent dye using a fluorescent microscope (Luckins and Mehlitz, 1978). An example of using IFAT in the screening of blood borne parasitic infections was the diagnosis of *Theileria equi* and *Babesia caballi* in domestic horses, southwest Mongolia (Ruegg *et al.*, 2007). The test was able to detect 78.8% and 65.7% of the examined animals to be infected with the two parasites, respectively.

1.3.4 Enzyme linked immunosorbent assay (ELISA)

Another important widely used immunodiagnostic test for the detection of antibodies or antigens of blood borne parasites diagnosis is ELISA; developed in 1971 by Engvall and Perlman for the measurements of antibodies for the diagnosis of different diseases (Engvall and Perlman, 1971).

Two types of ELISA have been developed depending on the target to be detected, the indirect ELISA is used for the detection of antibodies in a serum sample, based on the adhesion of a known antigen to the surface of a 96-well microtitre plate followed by the addition of the patient serum sample from which the antibodies will bind to the antigens if prior exposure has taken place. Finally, a second antibody labelled with an enzyme that catalyse the conversion of a colourless substrate to a visible coloured product are added (Engvall and Perlman, 1971; Voller *et al.*, 1976).

The other type is the direct ELISA such as antigen-ELISA or the double sandwich ELISA; used for antigen detection in which specific antibodies adhere to the surface of the microtitre plate. The sample to be examined is then applied and any antigen present is bound by the antibodies. Finally, the labelled antibodies with the enzyme are attached (Voller *et al.*, 1976). Figure 1.2 illustrates the indirect and sandwich direct ELISA.

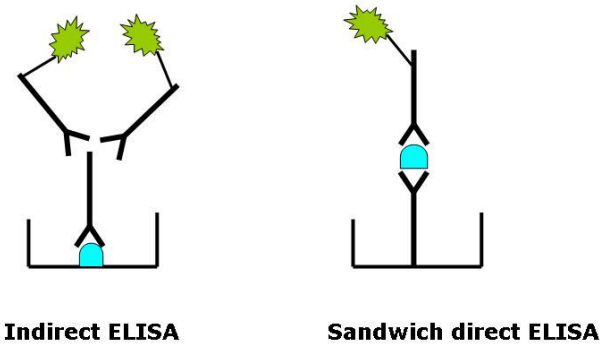


Figure 1.2: Indirect and sandwich direct ELISA

Ab-ELISA was used to detect anti-*Toxoplasma gondii* antibodies in 1258 small ruminants in Ghana and the results were compared to IFAT which was used as a reference test (van der Puije *et al.*, 2000). The ELISA test was found to be both highly sensitive (92%) and specific (91%) in the identification of the parasite antibodies in the examined animals. Similar sensitivity and specificity (90.2% and 92%, respectively) were also obtained using ELISA when comparing with IFAT in diagnosing *T. gondii* in pig serums from Ghana (Arko-Mensah *et al.*, 2000).

1.4 Molecular diagnosis

Molecular diagnostic testing is now firmly established as a routine diagnostic tool for infectious diseases. However, molecular tests have broader applications including use in epidemiological investigations, therapeutic strategies decisions and predicting the development of diseases (Caliendo and Dumler, 2001). Examples for the use of molecular tools in the diagnosis of blood borne parasitic diseases are fully discussed in section 1.5 of this chapter.

1.4.1 DNA hybridization

In eukaryotes, repeated Deoxyribo-nucleic acid (DNA) sequences known as “satellite DNA” have no known cellular function and typically contain a simple consensus sequence that is repeated thousands if not millions of times (Nantulya, 1991). These satellite repetitive DNA sequences give good sensitivity when used as hybridization probes for detection of complementary sequences in small amounts of test DNA.

The principle of DNA hybridization is that a single stranded DNA fragment containing the infectious agent species-specific sequences, is identified and purified, then labelled with a tracer (often radioisotope) and used to probe purified infectious agent DNA or whole organisms. Prior to application of the probe, the test DNA is treated with denaturing agents and split into single strands and immobilized on nitro-cellulose or nylon filters. When the probe is applied to the specimen, the sequences in the probe will hybridize with complementary DNA sequences (Figure, 1.3). The bound label can then be revealed by autoradiography (if the probe was labelled with a radioisotope).

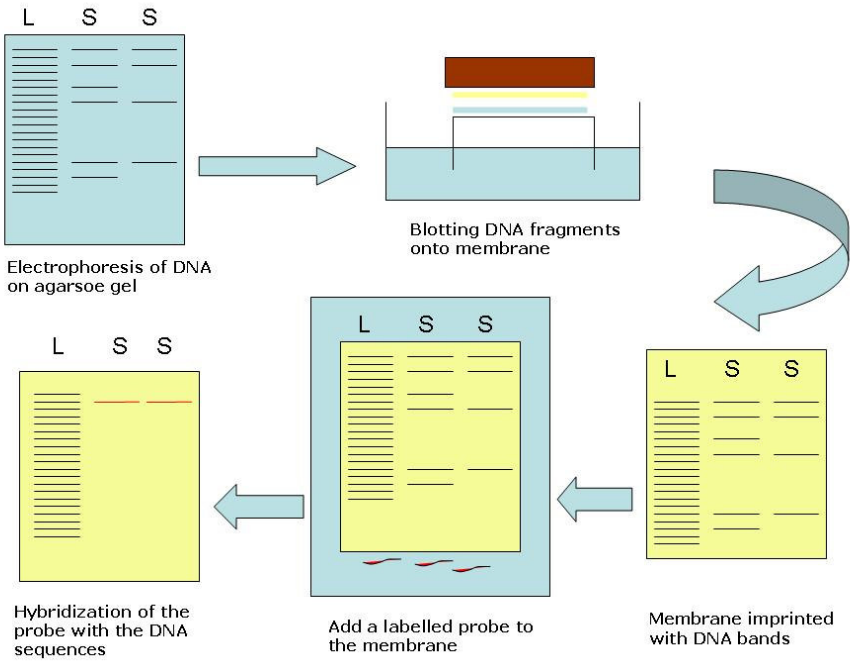


Figure 1.3: DNA hybridization using labelled probes (L: ladder, S: Samples), protocol adopted from Southern (1992)

Such procedures could form the basis of a diagnostic test because quantities of DNA down to 10-100 pg can be detected; this is equivalent to 10^2-10^3 protozoa per ml such as trypanosomes (Nantulya, 1991). The major constraint for field application of DNA probes are the preparation of specimens, hybridization of the probe and most importantly, the requirement of the radiolabel and the subsequent detection of the hybridization signal by autoradiography (Hide and Tait, 1991; Nantulya, 1991).

1.4.2 Restriction Fragment Length Polymorphism (RFLP)

Molecular methods have the advantage of the ability to examine DNA sequences which show variation between individuals or group of individuals using RFLP (Saiki *et al.*, 1985). The variation in DNA sequence in specific genes can be detected using a series of restriction enzymes which cut the

DNA at specific sequences. The variation is then assessed by examining the electrophoretically separated banding patterns of DNA digested with restriction enzymes after transfer onto nitrocellulose membrane using Southern blot technique followed by hybridization on the filter with a radio-labelled DNA probe. DNA polymorphism and variation can be detected by observing differences in banding patterns when comparing total DNA isolated from different organism stocks (Hide and Tait, 1991; Saiki *et al.*, 1985; Southern, 1992).

The limitation of this protocol is the low sensitivity especially if a single or low copy number gene is used for the assay and the use of Southern blotting which requires preparation of probes, radio-labelling and visualisation by autoradiography. Therefore, the use of RFLP has been used on the products from PCR in different fields for the study of the variation between individuals (Wolf *et al.*, 1999). PCR-RFLP was used for diagnosing filarial infection in 52 domestic cats in Thailand. Infection was demonstrated in 9.5% of the examined cats using PCR-RFLP which was able to detect two additional cases that were not detected using microscopy (Nuchprayoon *et al.*, 2006).

1.4.3 Nucleic acid amplification

Nucleic acid amplification is one of the most valuable tools in virtually all life science fields, including in field applications such as clinical medicine that requires diagnosis of infectious diseases (Notomi *et al.*, 2000). Several methods for amplifying target nucleic acid have been invented, such as Nucleic Acid Sequence Based Amplification (NASBA), Self-Sustained Sequence Replication (3SR), Strand Displacement Amplification (SDA) and PCR. PCR depends on heat denaturation of DNA to be subjected to the action of polymerase enzyme for amplification, whereas, the other three aforementioned protocols depend on another innovation to initiate new rounds of DNA synthesis. Briefly, NASBA and 3SR eliminate heat denaturation by using a set of transcription and reverse transcription reactions to amplify the target sequence (Compton, 1991; Guatelli *et al.*, 1990; Notomi *et al.*, 2000). While, SDA eliminates the heat denaturation step and employs a set of restriction enzyme digests and strand displacement for DNA synthesis with modified nucleotides as substrate. NASBA, 3SR and SDA methods lack specificity hindering them from being of wide scale use (Notomi *et al.*, 2000; Walker *et al.*, 1992).

PCR based assays currently constitute the main molecular diagnostic approach of researchers and health professionals. PCR allows the production of large quantities of a specific DNA from a complex DNA template in a simple enzymatic reaction; polymerases catalyse the synthesis of long polynucleotide chains using a single strand as a template (Newton and Graham, 1997). The hypothesis of replicating a piece of DNA using two primers was initially described by Kleppe *et al.* (1971), but progress was limited by primer synthesis and polymerase purification issues. In 1980s, Mullis and colleagues discovered a way to start and stop a polymerase based reaction at specific points along a single strand of DNA with the exponential amplification of the target sequence (Mullis *et al.*, 1986).

The basic amplification technique depends on an initial denaturation of the double stranded DNA at 95ºC for 3 minutes, followed by repeated cycles of denaturation, annealing and extension. Annealing of the synthesized primers to the specified regions in the target of interest occurs by decreasing the temperature to 50-60ºC. Extension of the target by activation of the polymerase at 72ºC utilises the annealed primers to synthesise a second strand of DNA on the single stranded template DNA (Figure 1.4). During this cycle, if the efficiency is perfect, the number of copies of the target DNA has doubled, depending on the number of the reaction cycles, the target DNA sequence can be amplified 2^n times where (n) is the number of reaction cycles (Hide and Tait, 1991; Newton and Graham, 1997). However, in reality, reactions do not maintain perfect efficiency because reactants within the PCR are consumed after many cycles, inactivation of polymerase and due to the self-annealing of the accumulating product after the copy number reaches high levels (Valasek and Repa, 2005).

In Mullis's original PCR process, *Escherichia coli* heat liable polymerase was used *in vitro*. The double stranded DNA was separated into two single strands by heating it to 95ºC leading to destruction of the enzyme, meaning that the enzyme had to be replenished after the heating stage of each cycle (Mullis *et al.*, 1986; Mullis and Faloona, 1987). In contrast, thermostable enzymes such as *Taq* polymerase which is derived from *Thermus aquaticus*, can be added at the beginning of the amplification process without further additions during the reaction (Newton and Graham, 1997).

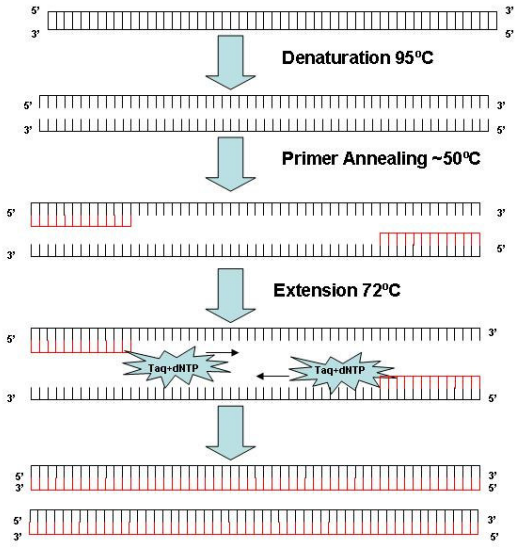


Figure 1.4: Diagram illustrating the basic PCR technique (protocol adopted from Newton and Graham (1997))

PCR based strategies have propelled molecular biology forward by enabling researchers to manipulate DNA easily (Valasek and Repa, 2005). Several distinct PCR formats are available that may be broadly be classified into ("high-tech", "mid-tech" and "low-tech") approaches (Reithinger and Dujardin, 2007). The most widely used approaches are those categorised as "Mid-tech", comprising conventional PCR assays in which PCR amplicons are resolved by electrophoresis and visualised after ethidium bromide staining. These assays are performed with several pieces of laboratory equipments such as thermocycler, power supply, electrphoresis tank, UV transilluminator and a camera (Reithinger and Dujardin, 2007; Singh *et al.*, 2005).

The category "High-tech" includes approaches in which PCR products are analysed during their amplification (real-time quantitative PCR, qPCR) utilising staining with a fluorescent dye (Reithinger and Dujardin, 2007). These assays are performed with a single all-in setup, and the detection of fluorescence is done within a closed tube decreasing the risk of contamination. Moreover, these applications are rapid and of high throughput, however, the equipment is comparatively expensive and the working costs remain high (Espy *et al.*, 2006; Reithinger and Dujardin, 2007). Quantitative PCR represents yet another technological leap forward that has opened up new and powerful applications for researchers throughout the world. This is in part because the enormous sensitivity of PCR has been coupled to the precision afforded by real-time monitoring of PCR products as they are generated. The basic goal of qPCR is to precisely distinguish and measure (quantify) specific nucleic acid sequences in a sample, even if there is only a very small quantity, by monitoring the amplification progress using fluorescent technology (Valasek and Repa, 2005).

Low-tech approaches refer to simplified PCR methods for use in laboratory settings with minimal equipments. Simplification can potentially be done at the two main steps of the PCR protocol that include target amplification and detection of the PCR products. Loop-mediated isothermal amplification (LAMP) represents a promising avenue for both steps requiring only a simple water bath for amplification and detection can be done visually by using SYBR Green I dye, which turns green in the presence of amplified products and remains orange in their absence (Reithinger and Dujardin, 2007).

LAMP was developed by Notomi *et al*. (2000) to amplify a few copies of DNA to 10^9 in less than an hour under isothermal conditions (constant temperature) and with high specificity. This method depends on auto-cycling strand displacement DNA synthesis using *Bacillus stearothermophilus* (*Bst*) DNA polymerase with high strand displacement activity. The products are a mixture of stem-loop DNAs with various sizes of stem and cauliflower-like structures with multiple loops. The main advantages of LAMP are the use of low-technology for the performance of the protocol especially in field conditions; this is mainly due to the use of a water bath because the reaction is performed at 65ºC. Additionally, LAMP was found to be highly sensitive and able to detect few copies of DNA (six copies) in the reaction mixture, the presence of non-target DNA in the reaction mixture had no significant effect on LAMP reactions. Moreover, visualization of the results can be done by noticing

turbidity or by using dyes such as SYBR Green I, making LAMP easier for practical in field use and less prone to contamination because the handling of the samples is minimized (Notomi *et al.*, 2000). Recently, the use of a real-time thermal cycler or real-time turbidimeter has been developed for the potential to quantify template DNA by the real-time monitoring of LAMP reaction using the increased intensity of fluorescence emitted from the used fluorescent reporter (Aoi *et al.*, 2006; Mori *et al.*, 2004).

1.5 Examples for the development of diagnostic strategies in three blood borne parasitic diseases

The main focus of this work is to study a variety of sample preparations (Chapters, III and IV) and molecular tools for the diagnosis of African trypanosomes (Chapter V). Trypanosomes are studied as a model for blood borne parasites, which occur in the blood stream as an extracellular parasite (outside blood cells). In the following section, the different diagnostic tools used for three other blood borne parasites are summarised. They were chosen based on their location either intracellular or extracellular in blood. *Plasmodium* was chosen as an example to an organism occurring intracellular within RBC, *Leishmania* was chosen as an example of an organism occurring intracellular within the white blood cells (WBC). Finally, *Trypanosoma cruzi* was used to exemplify organisms occurring extracellular or between blood cells. The aim of this literature search was to discuss specific sample preparation techniques used to detect each protozoon and to look at the different diagnostic approaches used for each organism.

1.5.1 Intracellular parasites in the red blood cells *(Plasmodium* species)

Malaria is a disease which can be transmitted to people of all ages through the bite of an infected mosquito. Approximately, 40% of the world's population, mostly those living in the world's poorest countries, are at risk of malaria with more than 500 million people becoming severely ill with malaria every year. The early diagnosis of malaria is the basic element for the control of this disease (WHO, 2007). The control, prophylaxis and eradication of this disease depends on the availability of sensitive, simple and inexpensive diagnostic means of detecting parasites in order to identify foci of infection and to evaluate the effect of various control programmes (Barker *et al.*, 1986). Although malaria is a frequently encountered disease in many developing countries, it is difficult to make the right diagnosis based on clinical signs; these include fever, headache, chills and vomiting. Such symptoms are not specific and could be found in many other diseases such as brucellosis and flu, so it is important to confirm the clinical suspicious using a good laboratory test in order to prevent unnecessary anti-malarial treatment (Mens *et al.*, 2007; WHO, 2007).

1.5.1.1 Diagnosis

There are four types of human malaria, *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. *P. falciparum* and *P. vivax* are the most common (WHO, 2007). Malaria cases caused by the most deadly type of malaria, *P. falciparum* have been estimated in 2004 to be within a range of 300-660 million cases (Snow *et al.*, 2005).

Direct microscopic examination of blood smears continues to be the method of choice (gold standard) for diagnosing acute malaria. This method is both sensitive and specific since malaria can be differentiated from other potential infectious agents, moreover, the various *Plasmodium* species can be readily distinguished. It is, however, time consuming to examine each slide, and a trained technician is required for accurate identification when low numbers of parasites are present (Barker *et al.*, 1986). Thick and thin blood films stained with *Giemsa* stain can be prepared for microscopy; thick blood films concentrate the layers of RBC on a small surface providing enhanced sensitivity for the detection of low levels of parasitaemia and reappearance of circulating parasites during infection recrudescence or relapse. Thin blood films (Figure 1.5) enable the fixation of RBC for morphological identification of the parasite to the species level and counting of the parasites to estimate the parasitaemia.

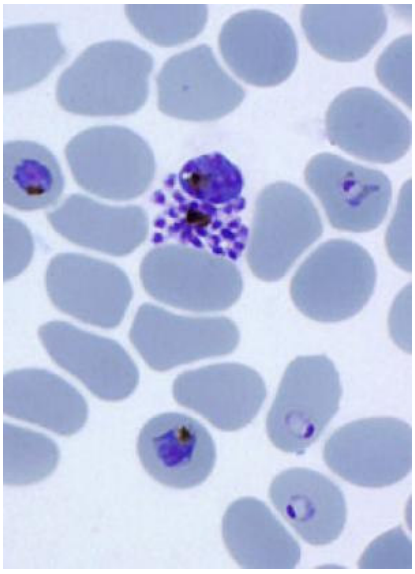


Figure 1.5: Human red blood cells infected with *Plasmodium falciparum*

(www.sciencedaily.com/images/2006/06/060620080915.jpg)

The sensitivity of blood films in diagnosing malaria was found to be 50 parasites/µl, although, examination of 100-400 microscopic fields is required before declaring the sample negative (Jonkman *et al.*, 1995; Mens *et al.*, 2007; Moody, 2002; Reyburn *et al.*, 2004). False positive diagnosis of

Plasmodium species using microscopy has been reported due to poor blood film preparation that generates artifacts leading to mistaken malaria diagnosis, these include bacteria, fungi, stain preparations, dirt and cell debris (Wongsrichanalai *et al.*, 2007).

Immunodiagnostic procedures such as ELISA for detecting malaria antibodies have been developed and tested providing important information with regard to exposure to malaria. The main drawback of this technique is the persistence of the antibodies after the disappearance of the malaria parasites from the blood following treatment, hindering the discrimination between present and past infections (Avraham *et al.*, 1982).

Assays depending on the detection of the parasite antigens have been developed to identify present infections; however, antibody for malaria antigen from a past infection may interfere with this assay causing false positive results (Avraham *et al.*, 1982; Barker *et al.*, 1986; Mackey *et al.*, 1982). The antigen based tests are termed by the World Health Organization (WHO) as Rapid Diagnostic Tests (RDT), they can detect 100 parasites/µl in a small amount of blood (5-15 µl) using an immuno-chromatographic assay with monoclonal antibodies impregnated on a test strip and directed against the target parasite antigen. Although the sensitivity of RDT remains a problem, the test is usually easy to perform without the need of any experience to interpret the coloured line on the test strip (Figure 1.6) and results can be obtained in five to twenty minutes without requiring capital investments or electricity (Wongsrichanalai *et al.*, 2007). However, parasite densities above 100 parasites/µl should be detected with confidence because the average parasitaemia seen in patients attending hospitals is between 50-5000 parasites/µl (Moody, 2002).

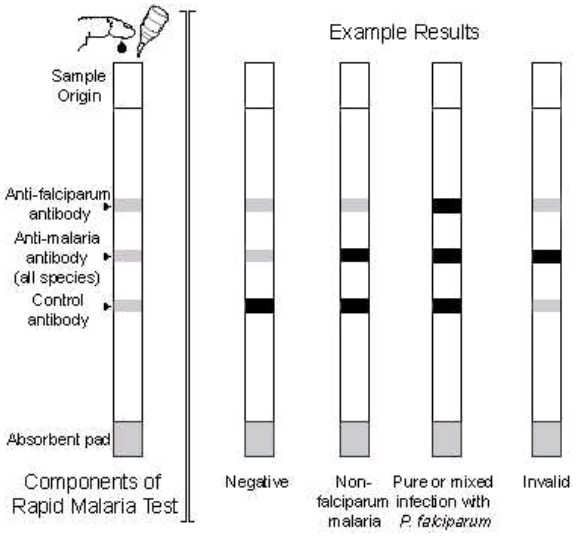


Figure 1.6: RDT test for malaria diagnosis (black line indicates positive results and grey line indicates negative results)

(www.fda.gov/Cber/blood/malaria071206sk9.gif)

The malaria antigens targeted by RDT are Histidine rich protein (HRT-1,2,3) and parasite specific lactate dehydrogenase enzyme (pLDH). These tests are not suited for follow-up after treatment because of the persistence of the antigens, therefore the specificity of these tests decreases (Mens *et al.*, 2007; Moody, 2002). The current market price of RDT in developing countries is ranging between US$0.55-US$1.50 compared with a range of US$0.12-US$0.40 per malaria smear to be examined by microscopy. Although, the cost of RDT is higher than microscopy, RDT is cost effective due to the rise in anti-malrial therapy costs that desperately requires accurate and rapid diagnosis (Wongsrichanalai *et al.*, 2007).

Diagnostic probes based on repetitive satellite DNA sequences are currently used for *Plasmodium* diagnosis. The probe is a DNA fragment containing a sequence of 21 bp that are repeated several thousand times in the malaria parasite genome. The technique is sensitive enough to detect 10 pg of purified *Plasmodium* DNA which is equivalent to 100 parasites. In the field, it has been observed that this technique can detect approximately 40 parasites/µl blood (Barker *et al.*, 1986). The advantages of this approach include the detection of current infection, results can be quantified and this method can be adapted to field use in blood samples obtained from a finger prick and applied directly to nitrocellulose paper without the need of DNA extraction (Barker *et al.*, 1986; Holmberg and Wigzell, 1987; Nantulya, 1991). In a study to evaluate the use of a DNA probe and microscopy for field diagnosis of malaria in Thailand, 20.4% of 632 patients were diagnosed to be positive using DNA probe, while, 19.1% were detected by microscopy. Subsequent examination of duplicate slides, from the cases diagnosed only with DNA probe, by expert microscopists confirmed that microscopy missed those cases (Wirth *et al.*, 1989).

The major advantage of using probes compared to microscopy, is the batch wise analysis of samples, meaning that one technician can examine 60 samples per day using microscopy, while, 1000 samples can be tested using probes per day (Barker et al., 1986). Another large scale study was conducted by Barker and colleagues (1994) in Thailand to examine the applicability of DNA probe-based method for in field diagnosis of *P. falciparum* compared with microscopy. The results revealed that between four and eight persons/day were required to obtain results from each set of 5000 samples by DNA probe analysis, whereas, microscopic examination required 150 persons/day. Approximate costs were US$ 0.17 per sample for DNA probe analysis, and US$ 0.36 for microscopic examination. In conclusion, the DNA probe method offers significant advantages when large numbers of samples must be surveyed (Barker *et al.*, 1994).

Quantitative nucleic acid sequence based amplification (QT-NASBA) can detect parasites at a level as low as 0.02 parasites/µl, and allow precise quantification of the parasite load in a range of 20-10^8 parasites/ml blood (Mens *et al.*, 2007; Schneider *et al.*, 2005). A cross sectional field study was reported by Ouedraogo *et al*. (2007) to validate the use of QT-NASBA in diagnosing and quantifying *P. falciparum* in 412 samples collected from Burkina Faso and comparing the sensitivity with

microscopy. The results revealed that the more sensitive QT-NASBA technique gave estimates of *P. falciparum* prevalence 3.3 fold higher than microscopy (Ouedraogo *et al.*, 2007).

PCR is said to have a detection limit of between 0.7 and 0.02 *Plasmodium* parasites/µl (Hänscheid and Grobusch, 2002; Hermsen *et al.*, 2001; Mens *et al.*, 2007). This reaction is used for the qualitative detection of the parasites regardless of the quantity of the infective species. A study conducted in a Brazilian amazon basin aimed to detect the prevalence of *P. malariae* using microscopy and PCR of the parasite after extracting the DNA from the blood sample applied onto filter paper. Microscopic examination of blood films showed prevalence of 1.2%, while, PCR revealed a prevalence of 11.9% (Scopel *et al.*, 2004).

In another study, PCR revealed 34.8% prevalence of malaria infected individuals in Lao People's Democratic Republic compared with only 17.3% detected by microscopy (Toma *et al.*, 2001). Moreover, PCR was especially sensitive for detection of 27 mixed infections with different malaria species but only two were detected by microscopy. In this study, extraction of DNA from 100 µl of heparinised blood using GFX (genomic blood DNA purification kits) were used as PCR template (Toma *et al.*, 2001). Another study conducted in Tanzania and Kenya, Mens *et al*. (2007) assessed the use of microscopy, RDT and molecular biology in malaria diagnosis. The authors confirmed the higher sensitivity of molecular tests including PCR and QT-NASBA in diagnosing malaria suspicious clinical cases. However, the drawback of using the molecular based techniques in diagnosing malaria is that they are expensive, complicated to perform and not widely available due to limited resources such as electricity and inadequate laboratory infrastructures in developing countries. Moreover, the impact of molecular tools in areas with low incidence is higher than in areas with higher malaria incidence (Hänscheid and Grobusch, 2002; Mens *et al.*, 2007).

Recently, qPCR has been developed and reported for quantification of low number of *Plasmodium* species, reaching 10 parasites per ml (Hermsen *et al.*, 2001). Although PCR based tools are sensitive, specific and able to detect very low parasitaemia, the costs and infrastructure needed to run and maintain PCR are not practical in most field settings where even intermittent electrical supply is a luxury (Boonma *et al.*, 2007).

Attempts for conducting simple and inexpensive molecular test to detect the highly conserved 18S ribosomal RNA gene of *P. falciparum* using LAMP technology have been described by Poon *et al*. (2006). The authors reported that the assay did not cross react with DNA from other *Plasmodium* species and indicated high specificity in diagnosing malaria when compared with PCR and microscopy. To reduce the cost and turnaround time of the LAMP test, the blood samples were prepared by heating at 99ºC for 10 minutes instead of utilising DNA extraction methods. More positive cases were reported using LAMP rather than PCR from the pre-heated samples, this might be attributed to the impact of proteins within this preparation having a greater inhibitory effect on the *Taq* polymerase of the PCR reaction. The LAMP test for the detection of *P. falciparum* is simple, cheap

and robust, moreover, there is no need for highly experienced staff to interpret the results, can be performed in the field with resource limited situations and not time consuming (Poon *et al.*, 2006).

Preparation of the samples prior to the application of PCR diagnostic methodology is an important issue to consider. In case of malaria parasites, the presence of the parasites intracellular in the RBC requires the release of the parasites from the cells and to remove the inhibitors that are present in blood. Distilled water was added to blood samples to lyse the blood cells in a study conducted in Nigeria for the detection of malaria using PCR (Uzoegwu and Onwurah, 2003). The use of DNA extraction kits on samples for malaria diagnosis was reported where lyses solution to lyse RBC releasing the malaria parasites is an essential step (Aslan *et al.*, 2007). Application of the blood sample onto filter paper and extraction of DNA using Chelex®100 was used in a large scale field study to characterise *P. falciparum* in the Republic of Cabo Verde using PCR (Alves *et al.*, 2006). The impact of lysing cells and using Chelex®100 in DNA extraction is fully discussed in Chapter VI.

1.5.2 Intracellular parasites in the WBC (*Leishmania*)

Leishmaniasis is a disease caused by *Leishmania* parasites, that are located intracellulary in macrophages (Figure 1.7), and transmitted to mammals including humans through the bite of phlebotomine sand flies. It is estimated that worldwide there are an annual 1.5 to 2 million cases with up to 350 million people at risk of infection.

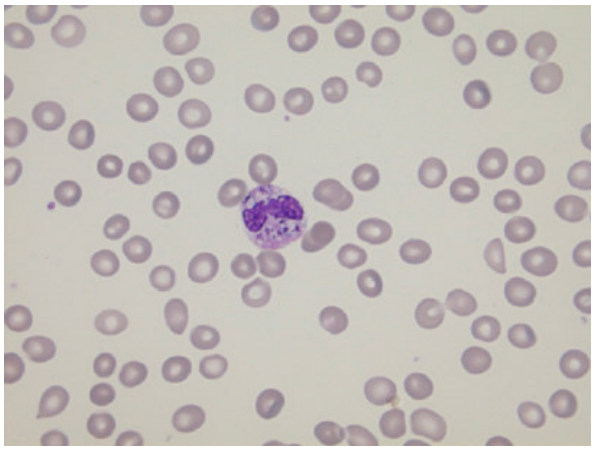


Figure 1.7: Blood film showing *Leishmania* intracellularly within macrophages

(www.nature.com/bmt/journal/v40/n4/images/1705728f2.jpg)

Diagnosis of leishmaniasis is considered a challenge because of the wide spectrum of clinical manifistations including, ulcerative skin lesions developing at the site of sand fly bite (localised cutaneous leishmaniasis), multiple non-ulcerative nodules (diffuse cutaneous form), destructive mucosal inflammation form and disseminated fatal visceral form (Murray *et al.*, 2005; Reithinger and Dujardin, 2007). Epidemiological studies carried out in the last few decades show that leishmaniasis is

a zoonotic disease because at least 15 *Leishmania* species (Table 1.1) found in mammals are infective for humans (Hide and Tait, 1991).

Table 1.1: *Leishmania* species and sub-species with the disease form caused (Hide and Tait, 1991)

Species	Sub-species	Disease form
Leishmania brasiliensis	*brasiliensis*	Muco-cutaneous form
	panamensis	Muco-cutaneous form
	guyanensis	Cutaneous
	peruviana	Cutaneous
Leishmania mexicana	*mexicana*	Cutaneous
	pifanoi	Cutaneous
	amazonensis	Cutaneous
Leishmania donovani	*chagasi*	Visceral
	donovani	Visceral
	infantum	Visceral
	archibaldi	Visceral
	sinensis	Visceral
Leishmania major		Cutaneous
Leishmania tropica		Cutaneous
Leishmania aethiopica		Cutaneous

1.5.2.1 Diagnosis

In recent decades, surveillance data indicates that the number of cases with leishmaniasis has increased; such an increase could be attributed to the improved diagnosis resulting in increased case reporting beside other factors such as inadequate vector control resulting in an absolute increase in disease incidence (Reithinger and Dujardin, 2007). The broad clinical spectrum of leishmaniasis makes the diagnosis more difficult and complicated, however, it should be accurate and differential because diseases caused by other etiological agents with clinical manifestations similar to those caused by *Leishmania* parasites are present at the same area such as skin diseases that might be diagnosed as cutaneous form (Reithinger and Dujardin, 2007). Moreover, the importance of accurate and differential diagnosis of leishmaniasis arises from the need to prescribe the highly toxic drugs only to patients affected with the more pathogenic species such as those causing the visceral form of the disease (Hide and Tait, 1991).

Microscopy of *Giemsa* stained smears (from blood, cutaneous lesion or visceral biopsy) is still the gold standard diagnostic approach in different health levels in endemic areas as more sophisticated techniques are currently expensive and rarely available (Herwaldt, 1999). The sensitivity of microscopy tends to be low and can be highly variable depending on the number and dispersion of parasites in biopsy samples, the sampling procedure and most importantly, the technical skills of the personnel (Herwaldt, 1999; Murray *et al.*, 2005; Reithinger and Dujardin, 2007).

Several serological approaches are commonly used in visceral form diagnosis, in particular, freeze-dried antigen-based direct agglutination tests and commercially available immunochromatographic dipstick tests due to their high sensitivity, ease of use and minimal laboratory and technological

expertise requirements (Chappuis *et al.*, 2006). However, serological tests are rarely used in cutaneous form because sensitivity can be variable and the number of circulating parasites tends to be low, as in cases of previous administration of chemotherapy. The specificity can also be variable, especially in areas where cross-reacting parasites such as *Trypanosoma cruzi* are prevalent (Chappuis *et al.*, 2006; Reithinger and Dujardin, 2007).

DNA hybridization technique has been found to be useful as a diagnostic procedure using labelled minicircle kDNA as a diagnostic probe (Nantulya, 1991). The minimum number of parasites detected by this technique was 10^2-10^3. Genomic DNA probes also exist but most are probes directed against single copy sequences, which are not generally suitable for diagnosis (Barker, 1987). Field application of DNA probes in diagnosing leishmaniasis was reported in Brazil for the identification of the parasites in 68 patients, of which, 94.1% belonged to *L. braziliensis* complex and 5.9% belonged to *L. mexicana* complex (Andrade *et al.*, 2005).

Practical applications of molecular methods for leishmaniasis diagnosis may be used to answer different clinical and biological questions (Reithinger and Dujardin, 2007). The first question is the detection of the parasite before initiating therapy; this includes analysis to the species or genus level. In this case, PCR has been shown to be better than microscopy, particularly in samples with low parasitaemia levels. Secondly, quantification of the parasite load may be highly relevant for monitoring disease progression and outcome of anti-Leshmanial therapy, qPCR with high analytical sensitivity reaching 0.0125 parasites per ml of blood has been reported with different *Leishmania* species. Although the higher sensitivity and accurate quantification achieved using qPCR, the cost of such tests remains higher than the conventional assays. It has been estimated that the cost per sample analysed using qPCR assays is $12, and $2.5 for analysis using PCR-RFLP assays (Reithinger and Dujardin, 2007).

The prevalence of leishmaniasis was studied using sero-diagnosis and PCR of blood samples collected from 100 dogs. The results revealed sero-prevalence of 26%, whilst, 63% were positive using PCR indicating higher sensitivity of PCR in diagnosing leishmaniasis (Solano-Gallego *et al.*, 2001).

Field samples collected for the diagnosis of the disease include cutaneous lesions, visceral biopsies and blood samples. PCR on blood samples for diagnosis depends mainly on the preparation of the blood sample. Extraction of DNA from blood was conventionally done using organic solvents (phenol-chloroform extraction), although this method is low cost if compared with commercial DNA extraction kits, it takes three hours to obtain the DNA (Alhassan *et al.*, 2007). Moreover, the organic solvents are known to persist in DNA extracts and can inhibit the PCR and require appropriate chemical disposal (Reithinger *et al.*, 2000).

Guanidine blood lysates have been used for lysing the blood cells to release the parasite from inside the macrophage cells (Reithinger *et al.*, 2000). Moreover, guanidine HCl is also suitable for storing

the samples at 4ºC or even at room temperature because it keeps the integrety of DNA for months. However, guanidine HCl might inhibit PCR because it is a salt, therefore, dilutions of extracted DNA might be required for successful amplification (Reithinger *et al.*, 2000).

In conclusion, compared to the standard microscopy and cell culture approach for *leishmania* diagnosis, the molecular approaches remain expensive and require technological expertise, and efforts should be made to make PCR simpler and cost-effective especially in remote areas where leishmaniasis is endemic (Reithinger and Dujardin, 2007).

1.5.3 Extracellular parasites (*Trypanosoma cruzi*)

The blood protozoa *Trypanosoma cruzi* (Figure 1.8) is the causative agent of Chagas' disease (American trypanosomiasis) which is transmitted to mammals by insect vectors called triatomine bugs (CDC, Fact sheet, 2007). These insects are commonly found in Latin America, especially in rural areas where poverty is widespread. The disease is named after the Brazilian physician Carlos Chagas who described the disease and the transmission cycle in 1909. The disease is acquired through contamination of the conjunctiva or any skin abrasion with the faeces of the infected bugs. The disease is the most important zoonotic parasitic disease in Latin America infecting more than 10 million people and about 50,000 chagasic patients die each year from the disease (CDC, Fact sheet, 2007; Miles *et al.*, 2003; Oelemann *et al.*, 1998).

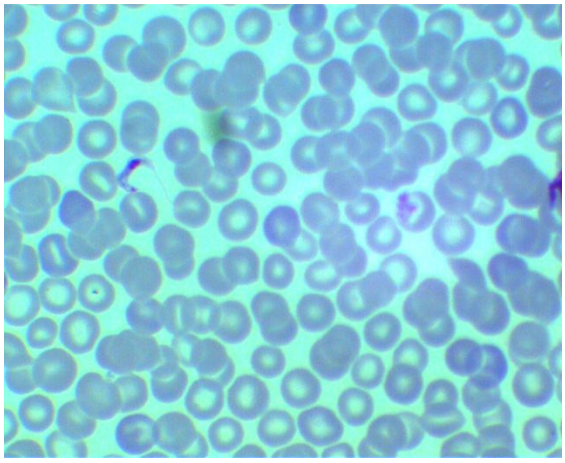


Figure 1.8: *T. cruzi* extracellular C- shape in blood film

(workforce.cup.edu/buckelew/Trypanosoma%20cruzi%20amastigotes%20in%20heart.htm)

The parasite is also transmitted through blood transfusion and congenital transmission as well as accidental ingestion of the vector faeces (Oelemann *et al.*, 1998). Clinically, at the site of exposure to infected faeces, local lesions in form of unilateral conjunctivitis and oedema (Romaña's sign) or cutaneous chagoma are developed. After the acute clinical manifestations disappear, the infection rests

with a long period of latency, called the indeterminate form period, which may last throughout life or evolve to a chronic phase (Chagas, 1922). The chronic phase is characterised by heart disease especially aneurysm of the apex of left ventricle because the parasite multiplies in heart muscle (Miles *et al.*, 2003; Tanowitz *et al.*, 1992). Gastrointestinal involvement also is a characteristic of the chronic phase.

1.5.3.1 Diagnosis

In areas where Chaga's disease is endemic, *T. cruzi* may infect as many as 74% of the general population (Pless *et al.*, 1992). Economic difficulties in such endemic areas are considered as a risk for the disease spread and the increased number of undiagnosed subclinical cases due to poor housing which is suitable for vector habitat (Garcia *et al.*, 1995). Therefore, sensitive, rapid and specific diagnostic assays are greatly needed for the acute Chagas' disease because early drug treatment of the acute form is presumed to be beneficial. The first approach for *T. cruzi* diagnosis is microscopic examination of blood films from suspicious patients (Kirchoff *et al.*, 1996). Xenodiagnosis, (meaning, in this context, feeding of laboratory bred triatomine on the patient followed by dissection of the insect looking for *T. cruzi*) and intensive blood cultures are used for diagnosing chronic phase patients due to low parasitaemia which is far below the threshold for microscopic detection (Miles *et al.*, 2003; Tanowitz *et al.*, 1992). Microscopic diagnosis of the vector hind gut is the gold standard method which is essential for vector control strategies; the usual standard for microscopic examination for parasite prevalence is based on the observation of 50 fields. The examination depends mainly on the detection of the parasite motility under the microscope which requires the examination of fresh samples (Pizarro *et al.*, 2007).

Serological assays such as IFAT, indirect hemagglutination, CFT and ELISA are used for diagnosis of *T. cruzi* infections due to their simplicity, low cost and good performance (Andersson, 2004). These tests depend on the detection of antibodies directed against the parasite; however, two tests should be used for the confirmation of the results because IFAT and hemagglutination often lead to false positive or false negative results due to antigenic cross reactivity with other parasites such as *Leishmania* and *Trypanosoma rangeli*, therefore ELISA is usually combined with these tests to confirm the results (Avila *et al.*, 1991; Garcia *et al.*, 1995; Oelemann *et al.*, 1998).

T. cruzi contains nuclear and kDNA, both of which contain many repetitive sequences that are highly suitable for PCR detection (Virreira *et al.*, 2003). A repetitive genomic DNA sequence has been used as a diagnostic probe (Nantulya, 1991). This probe consists of 195 bp repeating unit that constitutes 9% of the parasite genome. The sensitivity of this assay permits the detection of phenol-extracted DNA of 30 parasites genome equivalent from mammalian blood or tissues.

PCR based detection of *T. cruzi* from vector gut and from mammalian host blood is widely used for treatment and control purposes. Several primers complementary to the 330 bp conserved region of the

kinetoplast-minicircle part of the mitochondrial DNA have been used in PCR based assays (Moser *et al.*, 1989b). However, such PCR targeting the kDNA minicircle region require partial disruption of the dense kinetoplast minicircle with either a nuclease or by boiling the sample in the presence of strong chaotropic agent such as guanidine chloride to release the linear and amplifiable kDNA fragments. Moreover, the kDNA minicircle target has some sequence similarity to kDNA minicircle regions in other trypanosomatids such as *Leishmania* and other trypanosomes, decreasing the specificity of the target sequence. Primers amplifying a 188 bp repetitive nuclear sequence were found to be more sensitive in the parasite detection because this sequence was found in 1.8 times as many copies in *T. cruzi* genome as there are of the minicircle kDNA target, allowing the detection of low numbers of parasites. These primers amplify a target which is specific for *T. cruzi* parasites making it an ideal target for primer directed amplification of *T. cruzi* DNA without any pre-treatment of the sample (Moser *et al.*, 1989b; Pizarro *et al.*, 2007; Virreira *et al.*, 2003).

A study has been conducted to evaluate the performance of PCR amplification in the diagnosis of chronic Chagas' disease from whole blood and compared to serology (Avila *et al.*, 1993). The results indicated that the PCR amplification method has 100% sensitivity compared with serological testing. Nested PCR (the target DNA undergoes the first run of PCR using first set of primers, then the product from the first reaction undergoes a second run using a second set of primers) has also been designed for the amplification of flagellar protein gene (Tc-24) which directly demonstrates the presence of *T. cruzi* (Andersson, 2004; Vera-Cruz *et al.*, 2003). The authors argued that nested PCR is a good way to assess *T. cruzi* persistence because the second PCR step is considered as a confirmation that the appropriate parasite DNA is amplified.

A cross sectional study to determine the prevalence of *T. cruzi* infection among dogs in Oklahoma using PCR revealed that 3.6% of the examined dogs were infected (Bradley *et al.*, 2000). Another large field study has been performed in Bolivia with the aim of comparing PCR with other diagnostic methods for Chaga's disease. The results showed that PCR is highly sensitive when compared with ELISA and microscopy. The blood samples were lysed using guanidine to release the linear kDNA for amplification using PCR (Wincker *et al.*, 1997).

Quantitative PCR was developed by Cummings and Tarleton (2003) using SYBR Green I assay in order to demonstrate the utility of qPCR for quantitative measurement of tissue parasitic load in experimental *T. cruzi* infection. The authors optimised two specific primer sets, the first set that amplifies the tandemly repeated satellite DNA sequence exhibited greater sensitivity than the primer set amplifying kDNA (Cummings and Tarleton, 2003; Virreira *et al.*, 2006).

Piron *et al.* (2007) recently developed and evaluated the efficacy of qPCR assay to detect and quantify *T. cruzi* in blood of chagasic patients, using a TaqMan probe to guarantee the specificity of the measured signal. The qPCR is designed to amplify a 166 bp fragment in the satellite DNA of *T. cruzi* which has been shown to be specific, with a sensitivity of 7.8 down to 0.06 epimastigotes/ml. The

authors concluded that this new qPCR system was simpler, faster and more reliable than conventional PCR with a possibility of quantification and reduced risk of contamination.

LAMP reaction has been developed for the detection of *T. cruzi* using primer sets targeting the 18S rRNA gene (Thekisoe *et al.*, 2007). The primers have been shown to be highly specific because only the target DNA was amplified. Moreover, the sensitivity of the reaction was quantified using serial dilution of *T. cruzi* DNA starting from 10 ng down to 1 fg which is equivalent to DNA from 0.01 trypanosomes. The primers showed high sensitivity while detecting the protozoa DNA down to 1 fg, indicating that LAMP has the potential to be used as an alternative molecular diagnostic method especially at the under resourced laboratories because it takes only one hour for amplification, requires simple instruments (water bath) and it can produce large amounts of DNA that can be visualised by the naked eye because it depends on turbidity or colour detection (Thekisoe *et al.*, 2007).

1.6 Aims and outline of the thesis

The overall aim of the thesis is to investigate molecular diagnosis of trypanosomiasis as a model for blood borne infectious diseases. The approach adopted in the thesis is outlined below.

- Chapter II reviews the taxonomy and lifecycle of trypanosomes in vertebrate host and insect vector. Diagnosis of trypanosomes using different approaches starting from visualisation of the parasite by microscopy to different molecular tools is fully described.

- Chapter III validates the use of different sample preparations in the molecular diagnosis of trypanosome species.

- Chapter IV describes trials to improve the preparation of blood samples from paper based system (FTA®cards).

- Chapter V evaluates different PCR reactions in use for the molecular diagnosis of trypanosomes highlighting the advantages and drawbacks of each reaction.

- Chapter VI describes the application of the improved sample processing from FTA®cards and species-specific PCR reactions in field in a case study of the impact of the Stamp Out Sleeping sickness (SOS) mass treatment programme in the prevalence of different trypanosome species in new sleeping sickness foci, Uganda.

- Chapter VII aims to evaluate qPCR for quantifying *Trypanosom. brucei brucei* and *T. congolense* Savannah species in single and mixed infections of *Glossina morsitans morsitans*.

2 Chapter two

Diagnosis of African trypanosomiasis

2.1 Introduction

Trypanosomiasis is a disease occurring in Sub-Saharan Africa, caused by blood borne parasites of the genus *Trypanosoma* that are transmitted cyclically by *Glossina* species (Hoare, 1972). Trypanosomes have been reported in 38 African countries where they cause diseases of medical and veterinary importance (Masiga *et al*., 1996; WHO, 2001, , 2006). Human African trypanosomiasis (sleeping sickness) is caused by the parasites *T. brucei rhodesiense* and *T. brucei gambiense*; it is estimated that 300,000-500,000 people are currently infected, resulting in an estimated 100,000 deaths per annum (Cattand, 2001). However, the actual number is unknown because those infected with trypanosomes tend to live in areas with little or no medical care (El-Sayed *et al*., 2000). Moreover, sleeping sickness is likely to be highly under-reported because outbreaks tend to occur mainly among the rural poor and during civil strife when control measures are difficult to apply (Odiit *et al*., 2005).

It is estimated that three million cattle die annually from trypanosomiasis, *T. vivax* and *T. congolense* are regarded as major pathogens of cattle and other ruminants causing a disease called Nagana (Aksoy *et al*., 2003; Hoare, 1972). *T. brucei* affects all livestock, with particularly severe effects in equines and dogs, while, *T. simiae* causes high mortality in domestic pigs.

The greatest impact of animal trypanosomiasis is on the nutrition of millions of people living in the most highly endemic areas and on the agricultural economies of their countries as it renders vast areas of semi-arid savannah land in Africa unsuitable for raising domestic animals that are the source of dairy and meat products (El-Sayed *et al*., 2000).

2.1.1 Historical background

"It is tempting to believe that stages of a true trypanosome were first seen in 1680 by Antony van Leeuwenhoek (the Father of Protozoology) who described organisms seen by him in the gut of horse flies, which might have been the developmental stages of the bovine parasite *T. theileri.* However, serious attention to the trypanosomes of mammals was drawn by the work of Timothy Lewis (1878, 1879) on a parasite of Indian rats (*T. lewisi*). The discovery of the causative agent of Surra (*T. evansi*) in local horses and camels in India (1880) was the starting point of all subsequent investigations which incriminated trypanosomes in the aetiology of a series of diseases affecting man and livestock. The most important contribution to this subject was made by David Bruce (1880) who proved that the disease Nagana in livestock of Zululand was caused by a trypanosome (*T. brucei*) and also demonstrated that the disease was transmitted by tsetse flies and wild game animals acted as reservoirs of infection" (Hoare, 1972).

"The clinical signs of sleeping sickness were described during the 14th century in the historical writings of Al-Qalqashandi, who recorded that the Sultan of the Mali died from the disease in 1373-4 (Lumsden, 1974). Arab merchants and slave traders recognised the characteristic sign of the disease

(swollen cervical gland) making them reject slaves (Cattand, 2001). In 1902, trypanosomes were discovered in a human in West Africa by Dutton, and in the following year, similar trypanosomes were seen by Castellani (1903) in cerebro-spinal fluid (CSF) of a sleeping sickness patient in Uganda. Bruce had shown in 1903 that the mild infections observed in West Africa and sleeping sickness in East Africa were phases of the same disease. In 1909, the German investigator Kleine made the important discovery that *T. brucei* had to undergo cyclical development in tsetse flies (*Glossina*) before it became infective to a new mammalian host" (Hoare, 1972).

2.1.2 Structure of trypanosomes

Trypanosomes are blood borne parasites with a single nucleus, single flagellum and a leaf like or rounded body (Figure 2.1). The trypanosomes are members of the order Kinetoplastida characterised by the presence a kinetoplast (a DNA containing organelle) which is a part of the only mitochondria within the cell (Marquardt *et al*., 2000).

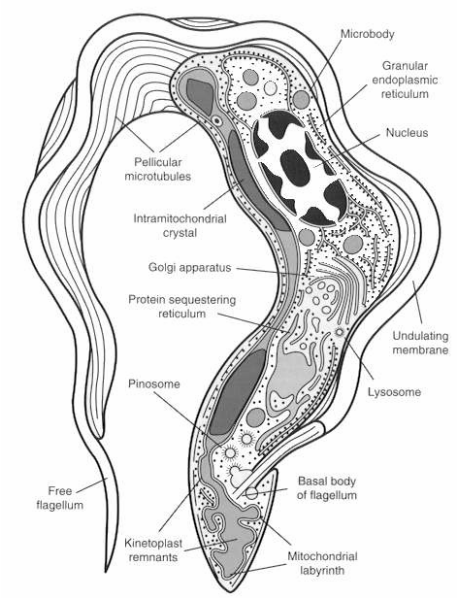


Figure 2.1: Trypanosome cell structure showing single flagellum and the kinetoplast (www.gsbs.utmb.edu/microbook/ch077.htm)

2.1.3 Taxonomy of trypanosomes

The genus *Trypanosoma* is divided into Stercoraria and Salivaria groups (Figure 2.2). The first group, the Stercoraria, trypanosomes develop in the hindgut of the vector and are then transmitted to the host through faecal contamination (the insect defecates after taking the blood meal). The second group, the Salivaria, the majority of trypanosomes are transmitted from the vector to the host during the act of blood feeding (Hoare, 1972).

For most species of Salivaria group, the vector for transmission is the tsetse fly. The two exceptions of this are *T. evansi* which is transmitted mechanically by tabanid flies, and *T. equiperdum* which is transmitted by direct sexual contact between equine hosts (Levine *et al*., 1980; Marquardt *et al*., 2000). Recently, Claes *et al*. (2005) hypothesized that some *T. equiperdum* strains are actually *T. b. brucei* or members of a subspecies of *T. brucei* (*T. b. equiperdum*) and that all other *T. equiperdum* strains are misidentified and are, in fact, *T. evansi*. This hypothesis was based on the existence of only small number of *T. equiperdum* laboratory strains after 100 years of research and the history of most of the strains is unknown. Moreover, the disease caused by *T. equiperdum* (dourine) has no definitive diagnosis based on serological or molecular methods, and only clinical signs are pathognomonic. The authors now propose to investigate their hypothesis by performing experimental infections in horses with *T. equiperdum* and comparing the produced clinical signs with the pathology of confirmed *T. b. brucei* and *T. evansi* strains (Claes *et al*., 2005).

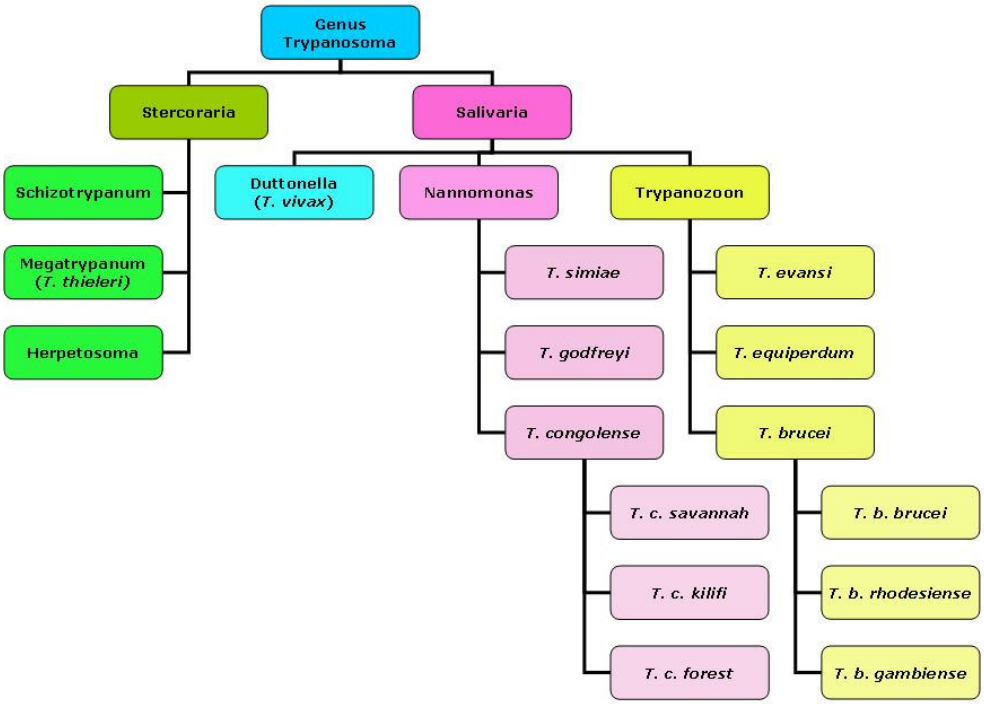


Figure 2.2: Taxonomy of trypanosomes (classification is based on Hoare (1972) and Marquardt *et al*. (2000))

2.1.4 Animal African Trypanosomiasis

2.1.4.1 Overview

The burden of animal trypanosomiasis is due to the direct losses resulting from mortality, morbidity and infertility of infected animals and the costs of controlling the disease (Hoare, 1972). Also, due to the indirect losses which include the exclusion of livestock and animal power-based crop production from the extensive tsetse flies infested areas (FAO, 1998,cited after (Kidanemariam *et al*., 2002)). It is estimated that three million cattle die annually from trypanosomiasis causing shortage in sources of animal protein. Therefore, the disease contidbutes to protein deficiency problems in tropical Africa (Marquardt *et al*., 2000).

2.1.4.2 Vertebrate host

Table 2.1 shows the host range of African trypanosomes in domestic animals. In the second column the list of livestock species is tentatively ranked in descending order of importance as an indication of pathogenicity (Uilenberg, 1998).

Table 2.1: Species of African trypanosomes with their hosts (Uilenberg, 1998)

Species	Domestic animal host	Reservoir
T. b. brucei	Horses, camels, dogs, sheep, goats, cattle, pigs	Several groups of wild mammals
T. b. rhodesiense	Horses, camels, dogs, sheep, goats, cattle, pigs	Several groups of wild mammals
T. b. gambiense	Horses, camels, dogs, sheep, goats, cattle, pigs	Several groups of wild mammals
T. congolense	Cattle, camels, horses, dogs, sheep, goats, pigs	Several groups of wild mammals
T. vivax	Cattle, sheep, goats, domestic buffaloes, horses	Several groups of wild mammals
T. simiae	Pigs	Warthog, bush pig
T. godfreyi	Pigs	Warthog

2.1.4.3 Life cycle

Trypanosomes transmission has two phases (Figure 2.3). One phase is inside the vertebrate host, while the other phase of development occurs inside the insect vector where cyclical development occurs (Uilenberg, 1998).

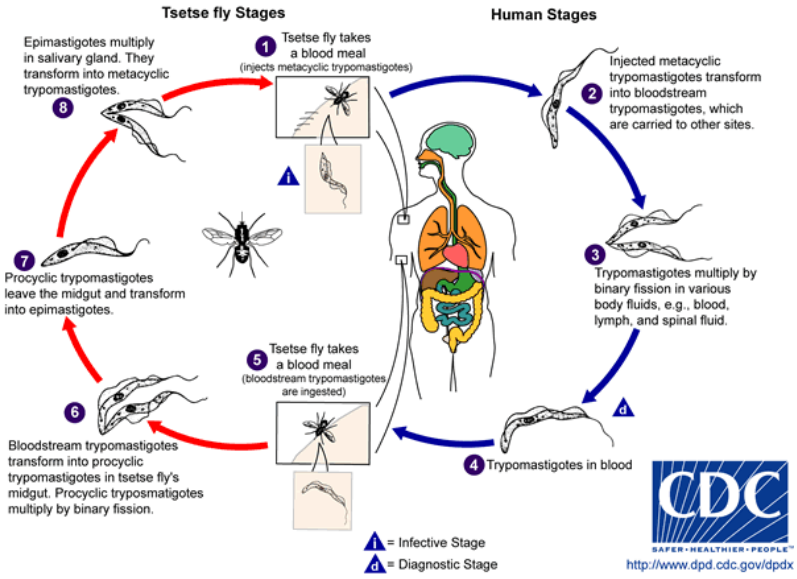


Figure 2.3: Life cycle of Trypanosomiasis in the vertebrate and invertebrate host

(www.dpd.cdc.gov/DPDX/HTML/ImageLibrary/SZ/TrypanosomiasisAfrican/body_TrypanosomiasisAfrican_il5.ht)

2.1.4.3.1 Inside the insect vector

The tsetse fly becomes infected with bloodstream trypomastigotes when taking a bloodmeal from an infected mammalian host. In the fly's midgut, the parasites transform into procyclic trypomastigotes that multiply by binary fission, leaving the midgut and transform into epimastigotes (Uilenberg, 1998). The epimastigotes reach the fly's salivary glands (in case of *Trypanozoon*) or mouthparts (in case of *Nannomonas*) and continue multiplication by binary fission giving rise to the infective metacyclics. In case of *Dutonella* species, trypanosomes mature in the proboscis of the fly but do not undergo a midgut maturation stage (Desquesnes and Dia, 2003). The period from ingesting infected blood to the appearance of the infective metacyclics varies according to the trypanosome species. It varies from five days in flies infected with *T. vivax* , two to three weeks in flies infected with *T. congolense*, while, three to five weeks is required in flies infected with *T. brucei* s.l (Vickerman *et al*., 1988).

2.1.4.3.2 Inside the vertebrate host

During a bloodmeal on the mammalian host, an infected tsetse fly injects metacyclics into skin tissue. The metacyclics undergo development and multiplication at the site of infection where a swelling or chancre may be detected in the skin. The mature bloodstream trypomastigotes are released via lymph vessels and lymph nodes into blood circulation. Trypomastigotes multiply by binary fission in various body fluids such as blood, lymph and spinal fluids (Uilenberg, 1998).

2.1.4.4 Factors affecting the disease severity

A number of host factors including physiological status of the host, nutritional and environmental factors have an important effect on the pathogenicity and determine the severity of the disease (Uilenberg, 1998). The pathogenicity of different species differs according to the host, for example, *T. vivax* is severely pathogenic to cattle, while *T. b. brucei* causes mild infection. The severity is also related to the geographical distribution, meaning that some East African isolates of *T. vivax* may cause an acute haemorrhagic disease in cattle, while, most West African *T. vivax* isolates cause a milder non-haemorrhagic disease. *T. congolense* is severely pathogenic to cattle and camels, while, moderately pathogenic to goats, sheep, horse and dogs (Taylor and Authie, 2004). Classically, in West Africa, acute *T. vivax* infection has been considered more important than *T. congolense*, whereas, in central or East Africa, *T. congolense* is considered the more important parasite (Eisler *et al*., 2004).

2.1.4.5 Pathogenesis

Infection of animals with *T. congolense*, *T. vivax* or *T. b. brucei* causes a disease known as Nagana, it is a chronic deblitating disease characterised by anaemia, roughness of hair coat, abortion, reduced milk yield, intermittent pyrexia, depression and gradual loss of condition (Figure 2.4), leading to extreme emaciation and death of the animal (Eisler *et al*., 2004; Hoare, 1972).

The lesion at the site of penetration is characterised by congestion and oedema. Plasma cells, lymphocytes and macrophages accumulate in this lesion then the parasites move into the lymphatic and circulatory systems (Uilenberg, 1998). Because of high parasitaemia, the body temperature rises to 39-41ºC, and the immune response of the body is directed toward the invading parasites leading to enlargement of the lymph nodes and the spleen.

Figure 2.4: Emaciated cattle with nagana

(www.dfid-ahp.org.uk/images/projectphotos/009-R8214.jpg)

Protection from the host immune response is afforded by variable surface glycoproteins (VSGs) which are glycosyl-phosphatidyl-inositol (GPI) anchored molecules that form a dense monolayer of identical

epitopes protecting against complement mediated lysis (Barry and Carrington, 2004). VSGs are only expressed in specialised telomeric loci called expression sites (ES) (Berriman *et al*., 2005). A small membrane portion of VSGs bearing limited antigen epitopes are exposed, these epitopes exhibit a high degree of variability between different VSGs. Meaning that switching VSG expression offers a mechanism to evade any antibody response that the host immune system may have developed. This mechanism is observed when waves of parasitaemia in infected patients occur due to the expression of different VSGs, resulting in the escape of small number of parasites from the host immune response (reviewed in Pays, 2006).

2.1.4.6 The zoonotic potential of trypanosomiasis

Animals are considered as an important source of food and transport for man. However, they are also an important source of many diseases that can be directly or indirectly transmitted to human (Seimenis, 1998). These diseases are zoonotic, and constitute a public health problem throughout the world (Coulibaly and Yameogo, 2000). The best agreed definition of zoonoses used by the WHO is that "they are those diseases and infections which are naturally transmitted between vertebrate animals and humans" (Shakespear, 2002).

The causative agents of zoonoses include prions, viruses, bacteria, protozoa, fungi and helminthes. It has been estimated that 62% of all human pathogens are classified as zoonotic (Taylor *et al*., 2001). It is well documented that *T. b. rhodesiense* is a zoonosis that is transmitted from a wide range of domestic and wild animals to human through the bite of tsetse flies. In particular, cattle are believed to be the important reservoir host for *T. b. rhodesiense* (Hide *et al*., 1996). In contrast, *T. b. gambiense* is not considered to be a zoonotic disease, however, recently a variety of wild animals and pigs were reported to harbour the pathogen (Njiokou *et al*., 2006; Simo *et al*., 2006)

2.1.5 Human African Trypanosomiasis

2.1.5.1 Overview

Human African Trypanosomiasis (HAT), known as sleeping sickness, remains a significant public health problem in sub-Saharan Africa. The disease has two forms, Gambian sleeping sickness (in central and western Africa) caused by *T. b. gambiense* and Rhodesian sleeping sickness (in eastern Africa) caused by *T. b. rhodesiense* resulting in chronic and acute forms, respectively (Apted, 1970). The obvious distinction of the two forms is that the Rhodesian form may be fatal within a few months due to central nervous system (CNS) involvement, damage to the heart and/or viscera, whereas the Gambian form may progress for years before death due to central nervous system damage. Sleeping sickness is placed third behind malaria and schistosomiasis in terms of the burden of parasitic diseases in sub-Saharan Africa (Cattand, 2001).

The incidence of *T. b. rhodesiense* sleeping sickness is characterised by short epidemics interspersed between long periods of low undetectable endemicity (Hide *et al*., 1996). One possible mechanism for the generation of an epidemic is the introduction of new strains of parasites which may be more virulent or have a higher level of transmissibility through the human population. The possible explanations for the introduction of such new strains could be the change in the ecology resulting in a higher transmission of existing human infective trypanosomes from reservoir hosts to humans, genetic exchange or the appearance of new strains from other regions (Hide *et al*., 1996).

2.1.5.2 Disease stages

2.1.5.2.1 First stage

Human African Trypanosomiasis is characterised by two stages; the first stage is the haemolymphatic stage which corresponds to the invasion of lymph, blood and other tissues by trypanosomes. At the site of the fly bite, a trypanosomal chancre (Figure 2.5) may appear after 5-15 days of infection, other early signs include itching, erythema, swelling and pain. After three to four weeks, the chancre usually heals with overlying desquamation. Invasion of the blood stream by the trypanosomes leads to fever, headache and general malaise.

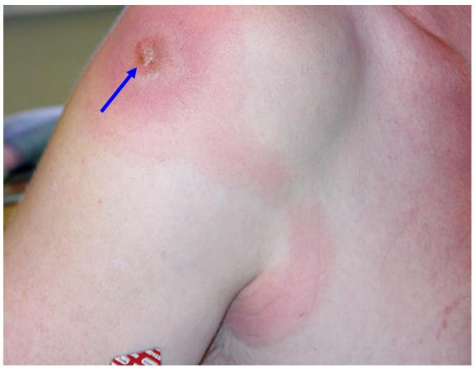


Figure 2.5: Trypanosomal chancre at the site of the fly bite

(www.cdc.gov/ncidod/eid/vol8no1/images/01-0130ab.jpg)

Other clinical signs include adenopathy especially enlarged neck gland causing Winterbottom's sign (only in case of Gambian form) (Figure 2.6); skin rash, oedematous swelling of the lower eyelids and puffy swollen appearance of the face. Moreover, cardiovascular disturbances such as arrhythmia and hypotension are early signs of the acute phase (Barrett *et al*., 2003; Buscher and Lejon, 2004).

Figure 2.6: Winterbottom's sign (enlarged neck gland in Gambian sleeping sickness)

(commons.wikimedia.org/wiki/Image:Winterbottom's_sign.jpg)

2.1.5.2.2 Second stage

The second stage is the meningo-encephalitic stage, which occurs due to crossing of the blood brain barrier by the parasite. This stage occurs within a few weeks of infection with *T. b. rhodesiense* but over a period lasting between several months or years with *T. b. gambiense*. Signs of nervous system injury become more obvious especially in the Gambian form due to long duration of the disease. As this stage progresses, headaches become severe and sleep disorders begin to manifest. This stage is also characterised by disorders of tonus, motility and abnormal movement behaviour. However, in the Rhodesian form the marked neurological symptoms are often not clear because death occurs more rapidly (Barrett *et al*., 2003; Buscher and Lejon, 2004).

2.1.6 Vector

2.1.6.1 Introduction

African trypanosomes are transmitted between vertebrate hosts by tsetse flies that belong to genus *Glossina* of family *Glossinidae* (Figure 2.7). Tsetse flies are restricted to sub-Saharan Africa between latitudes 5^{o}N and 20^{o}S in an area of 8.5 million km^{2} which represents more than 40% of the total land area of 38 African countries (Allsopp, 2001), except for a report early in the 20th century from the Arabian Peninsula where small tsetse pockets were reported in the southwest part (Barrett *et al*., 2003). They breed along different habitates such as rivers, streams and bush; being active during the day and feed exclusively on blood. Unlike most biting flies, where only females are bloodsuckers, both male and female tsetse flies require bloodmeals. The fly is characterised by the wings overlaping one another at rest and the presence of "a hatchet cell" in the centre of the wing (Marquardt *et al.*,

2000; Uilenberg, 1998). After trypanosomes develop in the tsetse host, the fly remains infective over its life span, which is around five to six weeks in the field (Welburn and Maudlin, 1999).

Figure 2.7: Tsetse fly feeding on blood

(www.dfid-ahp.org.uk/images/gallery/target133.html)

2.1.6.2 Taxonomy of the vector

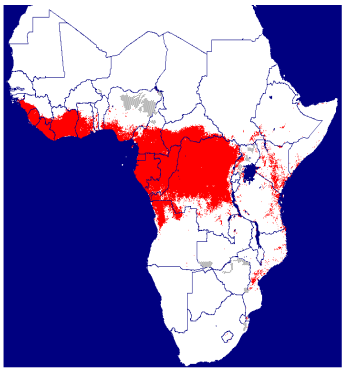
Tsetse have been divided into three sub-genera, *Glossina*, *Nemorhina* and *Austenina*, that are commonly called *morsitans*, *palpalis* and *fusca* groups, respectively (Pepin and Meda, 2001). The species and subspecies are listed in Table 2.2.

Table 2.2: Taxonomy of the vector (Rogers and Robinson, 2004; Uilenberg, 1998)

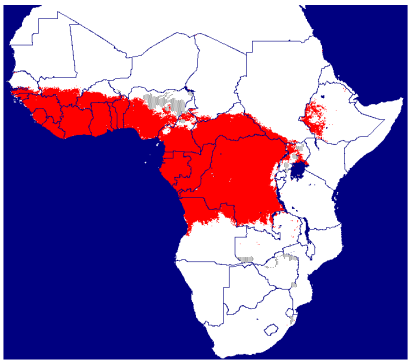
Morsitans group		Palpalis group		Fusca group	
Species	**Sub-species**	**Species**	**Sub-species**	**Species**	**Sub-species**
G. morsitans	*G. m. morsitans*	*G. palpalis*	*G. p. palpalis*	*G. fusca*	*G. f. fusca*
	G. m. submorsitans		*G. p. gambiensis*		*G. fusca congolense*
	G. m. centralis	*G. fuscipes*	*G. f. fuscipes*	*G. nigrofusca*	*G. n. nigrofusca*
G. pallidipes			*G. f. martini*		*G. n. hopkinsi*
G. swynnertoni			*G. f. quanzensis*	*G. brevipalpis*	
G. austeni		*G. pallicera*	*G. p. pallicera*	*G. fuscipleuris*	
G. longipalpis			*G. p. newsteadi*	*G. haningtoni*	
		G. caliginea		*G. longipennis*	
		G. tachinoides		*G. medicorum*	
				G. nashi	
				G. severini	
				G. schwetzi	
				G. tabaniformis	
				G. vanhoofi	
				G. frezili	

2.1.6.3 Distribution

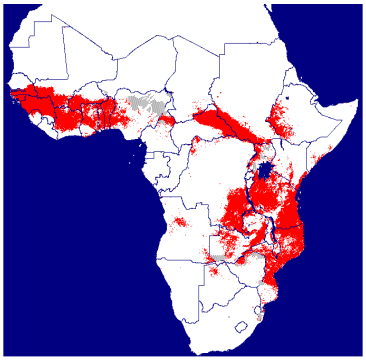
The classification of tsetse is based on morphological criteria dividing the species into the aforementioned three groups. The geographical distribution of these groups is shown in Figure 2.8. In summary, the fusca group flies tend to occur in the lowland rainforests of West and Central Africa (Figure 2.8a). While, palpalis group flies occupy similar forest habitats throughout Africa and extend into riverine and lakeside forests or the moist areas between such forests (Figure 2.8b). Finally, the morsitans group of flies occurs in a variety of savannah habitats lying between the forest edges and deserts (Figure 2.8c) (Rogers and Robinson, 2004).



a) Distribution of fusca group



b) Distribution of palpalis group



c) Distribution of morsitans group

Figure 2.8: Distribution of tsetse groups

(http://ergodd.zoo.ox.ac.uk/livatl2/tsetse.htm)

Host preference of the vector was investigated by Weitz (1963) who analysed over 22,000 tsetse blood meals collected from all over Africa using precipitin test. The authors categorised the tsetse according to their feeding preference in to five groups as shown in Table 2.3.

Table 2.3: Feeding preference of *Glossina* species (Uilenberg, 1998)

Group	Glossina species	Host preferred
1	*G. swynnertoni, G. austeni, G. tabaniformis and G. fuscipleuris*	Mainly Suidae
2	*G. m. morsitans and G. m. submorsitans*	Equally feed on Suidae and Bovidae
3	*G. pallidipes, G. longipalpis and G. fusca*	Mainly Bovidae
4	*G. brevipalpis and G. longipennis*	Mainly mammalian species other than Suidae and Bovidae
5	*G. p. palpalis, G. f. fuscipes and G. tachinoides*	Most available hosts and man

2.1.7 Control

2.1.7.1 Tsetse control strategies

Vector control is considered an important approach to control both human and animal trypanosomiasis. The female fly produces a single egg over a period of 12-14 days that develops inside the uterus into a larva that weighs more than the female (Hargrove, 2004). This means that a female produces no more than eight to 10 eggs in a lifetime of 14 weeks. This low reproductive rate allows the control programmes targeting the flies to be more effective resulting in reduction of the vector number.

Until the development of organochlorine insecticides such as Dichlorodiphenylethans (DDT) in 1940s, the principle means of tsetse control were the extermination of game and the destruction of bush (Swynnerton, 1921). Although these methods have been used successfully in the past to control tsetse, they are no longer used due to their ecological implications (Grant, 2001).

The application of insecticides can be done from air or from the ground. Ground application aims to apply a deposit of residual insecticides to resting sites of tsetse on vegetation where it remains lethal to tsetse beyond the maximum pupal period of about 60 days (Holmes, 1997). This method is the only proven method of large-scale tsetse eradication, for example the elimination of tsetse from 200,000 km^2 of Nigeria (Jordan, 1978). In the past, DDT has been commonly used; however, the use of residual insecticides is now environmentally and politically unacceptable. Recently, pyrethroids such as deltamethrin (Vectocid®) have been applied using the ground application method. Problems have been encountered with this method such as developing adequate access, maintaining a fleet of vehicles and spraying items, and supervising large numbers of spray teams (Holmes, 1997).

In order to overcome the drawbacks of ground spraying, aerial spraying was increasingly adopted during the 1970s and 1980s using the sequential aerosol technique. This approach applies very low doses of insecticides as aerosols either from low flying aircraft or helicopters at intervals timed to kill newly emerged flies before they could reproduce to deposit larvae in the soil. The sequential application of five sprayings is found to be sufficient and has no residual effect due to the very low doses used. This method has advantages over ground spraying as a result of reduced labour demands

and the centralisation of activities and support facilities. However, it is expensive and requires substantial funding at national and international level (Grant, 2001; Holmes, 1997).

In the early 1970s, bi-conical traps (Figure 2.9) made from blue and black materials that attract tsetse flies, were developed and successfully used against riverine species such as *G. palpalis palpalis* (Allsopp, 1984). However, a study conducted at the same time in Zimbabwe, demonstrated that the traps failed to catch many of the tsetse that visited them and most of the tsetse were instead attracted to a stationary animal in response to the host's odour (Vale, 1980). Therefore, different attractants such as carbon dioxide, acetone, butanone, 4-methylphenol and 3-n propylphenol have been used to attract tsetse flies to the traps. The use of the aforementioned attractants increased the catch of the trap by 20-fold for some species of tsetse. Moreover, cheaper and simpler targets have been developed, they are insecticide treated cloth screens baited with synthetic odours. The flies come in contact with the target and pick up a lethal dose of insecticide. Targets have the advantage of being relatively more effective and cheaper than traps, although traps have the distinct advantage of displaying dead tsetse flies, which helps to motivate local people to participate in schemes using this technique (Grant, 2001; Holmes, 1997).

Figure 2.9: Bi-conical tsetse traps

(www.nri.org/tsetse/FAQ/Pics/ph_biconique2.jpg)

In recent years, the use of insecticide sprays, dips or pour-on for cattle, have been applied as live bait application in areas infested with tsetse flies. Pyrethroides are the chemical of choice for this application, the scheme has been proved to be successful and major reduction in tsetse populations have been achieved in different areas such as Tanzania, Burkina Faso and Ethiopia (Bauer *et al*., 1995; Fox *et al*., 1993; Holmes, 1997; Rowlands *et al*., 2001).

Another method for tsetse control is the use of sterile insect technique (SIT) that depends on the release of irradiated sterile males into the environment. The sterile males mate normally with females

preventing them from producing any offspring resulting in reduction of the tsetse population (Hargrove and Williams, 1998). This technique has been successfully used in the island of Zanzibar, although the situation in Zanzibar differs than the other African countries because this island is small and geographically isolated from other countries reducing the chance of re-infestation from neighbouring areas (Bailey, 1998; Vale and Terr, 2004).

2.1.7.2 Drugs

2.1.7.2.1 Treating the animal host

The application of drugs has been the main control strategy against trypanosomiasis for more than 40 years. There are currently only three trypanocides available for controlling tsetse transmitted trypanosomiasis in animals. These drugs are isometamedium chloride (trade name; Samorin®, Trypamidium®, Veridium®) and homidium (chloride salt; Novidium®; bromide salt or ethidium bromide; Ethidium®), both having curative and prophylactic effect. The third drug is diminazene aceturate (trade names; Azidine®, Berenil®, and Veriben®) has only therapeutic properties (Browning *et al*., 1938).

Isometamedium chloride and homidium are phenanthridinium compounds, whose antitrypanosomal activity was first demonstrated in 1938. Their mode of action is mainly blockage of nucleic acid synthesis through intercalation between DNA base pairs and inhibition of DNA and RNA polymerases (Browning *et al*., 1938; Kinabo, 1993). Both isometamedium chloride and homidium are active against *T. congolense* and *T. vivax*, moreover, isometamedium is of value against infections caused by *T. brucei* s.l (Kinabo, 1993). Isometamidium is routinely administered intra muscular (i.m) at doses of 0.25-0.5 mg/kg for therapeutic purposes, and 0.5-1 mg/kg for prophylactic purposes for two to three months, with a maximum dose for resistant cases of 2 mg/kg. Homidium is recommended for i.m use at a dose of 1 mg/kg (Kinabo, 1993; Sinyangwe *et al.*, 2004). The recommended dose of diminazene aceturate is 3.5 mg/kg by i.m route, however, twice this dosage is recommended for *T. brucei* s.l. infections. The trypanocidal activity of diminazene aceturate is mainly due to its ability to inhibit kDNA replication and cause alterations in ribosomes, cytoplasmic membranes and amino-acid transport mechanisms (Peregrine and Mamman, 1993).

The emergence of drug resistant trypanosomes has been reported in many African countries (Geerts *et al*., 2001). It is estimated that 35 million doses of trypanocide drugs are used in Africa each year with about 50-70 million animals at risk from trypanosomiasis (Geerts and Holmes, 1998). In the past, the availability of trypanocides was strictly controlled in most African countries by Government Veterinary Departments. However, recently, with the privatization of veterinary services and a general trend towards deregulation of markets, trypanocides have become widely available increasing the risk of drug resistant trypanosomes emergence (Geerts *et al*., 2001).

2.1.7.2.2 Treating the human cases

The type of treatment depends on the stage of the disease. In the first stage of the disease, the drug used is less toxic, easier to administer and more effective. Treatment success in the second stage depends on a drug that can cross the blood-brain barrier to reach the parasite; such drug is quite toxic and complicated to administer (WHO, 2006). There are four drugs available for treating human African trypanosomiasis; these include Pentamidine, Suramin, Melarsoprol and Eflornithine. Most of these drugs are toxic and encounter parasite resistance (Docampo and Moreno, 2003). With the exception of Eflornithine (registered in 1990) no new drugs have been developed for sleeping sickness in 60 years (Pepin and Meda, 2001). Table 2.4 summarizes the use, application and dosage of those drugs (Burchmore *et al*., 2002; Docampo and Moreno, 2003).

Table 2.4: Drugs used against Human African Trypanosomiasis (Burchmore *et al.*, 2002; Docampo and Moreno, 2003)

Drug	**Pentamidine**	**Suramin**	**Melarsoprol**	**Eflornithine**
Chemistry	**Diamidine**	**Sulphated naphthylamine**	**Arsenical**	**Difluoromethylornithine**
Solubility	Water soluble	Water soluble	Poorly soluble in water, ether or alcohol	
Application	I/M	Repeated slow I/V injections	I/V	I/V
Indications	Early stage *T.b.gambiense*	Early stage *T.b.rhodesiense* *T.b.gambiense*	Early & Late stage *T.b.rhodesiense* *T.b.gambiense*	Early & Late stage *T.b.gambiense*
Dosage	4mg/kg body wt* once daily for 7 days	5mg/kg body wt on day 1 followed by 5 injections of 20mg/kg body wt on days 3, 10, 17, 24 and 31.	3-4 daily injections of 3.6mg/kg body wt separated by rest periods of 7-10 days	400mg/kg body wt (100mg every 6 hours) for 14 days
Side effect	Injection sites are very tender and sterile gluteal abscesses may occur	-Mild renal toxicity -I/M or S/C injections cause inflammatory response	Highly toxic	
Notes	Poor penetration to CNS	Poor penetration CNS		-expensive -trypanostatic rather than trypanocidal

*** Weight**

2.2 Diagnosis

Rapid and accurate diagnosis is necessary in any clinical conditions. In the context of animal and human trypanosomiasis in the field, the rapidity and accuracy of any technique or a combination of techniques is vital if the treatment of any individual is to be effective, or if chemotherapeutic control of a selected group of animals is to be attempted (Molyneux, 1975). The primary reason for trypanosomiasis diagnosis is the appropriate application of therapeutic and sometimes prophylactic measures. In order to design rational control strategies and for research purposes, knowledge of the epidemiology of the disease is imperative, which in turn depends on the sensitivity and specificity of the selected methods of trypanosome diagnosis (Hutchinson *et al*., 2003).

2.2.1 Clinical diagnosis

Diagnosis of trypanosomiasis on the basis of clinical signs is difficult because there are no specific pathognomonic symptoms. The clinical signs in humans (section, 2.1.5.2) could be mis-diagnosed with other diseases causing cutaneous lesions, such as leishmaniasis; however, the pathognomonic disturbance of the sleep is usually obvious at late stage of the disease, where cure from the disease becomes more complicated. In animals (section, 2.1.4.5) the clinical signs are not specific and could be mis-diagnosed with other diseases such as fasciolosis, schistosomiasis and haemonchosis (Catley *et al*., 2001).

2.2.2 Parasitological diagnosis

Microscopical examination of wet or *Geimsa* stained thin and thick blood films (Figure 2.10) is considered the traditional method for trypanosome diagnosis in the field. Although being widely used, these techniques are not considered sensitive enough to detect the low parasite levels especially in chronic cases. Therefore, these methods have been modified using concentration techniques to improve the sensitivity of the microscopic based methods (Luckins, 1977, , 1992).

The most commonly used concentration method is the haematocrit centrifugation technique (HCT) developed by Woo (1970) using centrifugation of microhaematocrit capillary tubes containing the blood sample and examination of the buffy coat/plasma junction (Figure 2.11) under the microscope. In a positive diagnosis, trypanosomes are found wriggling at the junction of the buffy layer and the plasma (Woo, 1970).

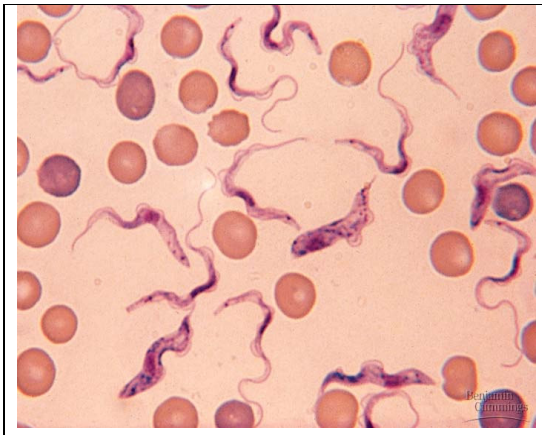


Figure 2.10: Trypanosomes in blood film

(io.uwinnipeg.ca/~simmons/16cm05/1116/28-11-TrypanosomaLM.jpg)

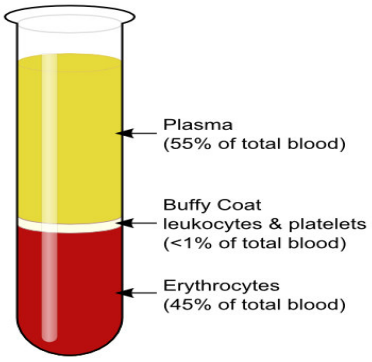


Figure 2.11: Blood components after centrifugation

(en.wikipedia.org/wiki/Image:Blood-centrifugation-scheme.png)

Improvement of the HCT was achieved by cutting the capillary tube 1 mm below the buffy coat and expressing the buffy coat/plasma interface on a microscope slide for examination using dark-ground or phase contrast microscopy (Murray, 1977). This method is known as the buffy coat technique (BCT). A biproduct of this approach is the calculation of the packed red cell volume (PCV) in advance to buffy coat aspirationas. The PCV can be used as a measure of anaemia as a useful indicator of trypanosome infection.

Parasitological techniques commonly applied in the detection of trypanosome infections have inherently poor sensitivity, and therefore fail to detect a high proportion of chronic and often aparasitemic infections (Masake *et al*., 2002). Even using techniques to concentrate the parasite for microscopical diagnosis, the sensitivity is still low. Paris *et al*. (1982) compared the use of parasitological techniques for the diagnosis of African trypanosomiasis in cattle and found that the BCT was the most sensitive followed by HCT, *Giemsa* stained thick blood film; *Giemsa* stained thin blood film and then wet blood films. The analytical sensitivity of the BCT in detecting trypanosomes was found to be $2.5x10^2$, $5x10^2$ and $5x10^3$ for *T. congolense*, *T. vivax* and *T. brucei*, respectively (Eisler *et al*., 2004; Paris *et al*., 1982). Furthermore, the sensitivity of HCT and BCT for *T. vivax* detection using microscopy was estimated by Desquences (1997) to be at 10^2-10^3 trypanosomes/ml blood.

Application of parasitological techniques in field has been widely used for the diagnosis of African trypanosomiasis in both the vertebrate (domestic and wildlife) and invertebrate hosts. Blood samples from randomly selected dairy and beef cattle from Tanzania were examined for the presence of different trypanosome species. Fifty two animals (13.3%) were diagnosed as harbouring trypanosomes by examining wet, thick and thin blood films prepared from buffy coat content after centrifugation of the blood sample (Mugittu *et al*., 2001). Goossens *et al*. (2006) screened blood samples collected from cattle population of Mafia Island, Tanzania, using BCT. Microscopy revealed that 3.9% of the examined animals were infected with trypanosomes. Wildlife has been found to be infected with trypanosomes (38.3%), in a study conduced in Tanzania by Geigy *et al*. (1971) using examination of thick and thin blood smears. While Majiwa *et al*. (1994) examined 3044 flies caught from Kenya and Uganda for the natural infection with trypanosome species using microscopy. The results revealed that 156 (5.1%) flies were infected with trypanosomes. The identified species were 71 *Nannomonas* type or immature *Trypanozoon* type (gut and mouthparts) infections and 85 had *T. vivax* type (mouthparts only) infections.

2.2.3 Sub-inoculation methods

Sub-inoculation refers to methods where trypanosomes are transferred from a suspected case by inoculating the blood into another susceptible animal, invertebrate host or *in vitro* culture system.

Rodent sub-inoculation

Rodent sub-inoculation has been widely used for diagnosis and preparation of trypanosome stabilates. Immunosuppressants (such as cyclophosphamide) are used to increase the susceptibility of the rodents to infections. This method has been found to be more effective for trypanosomes in *Trypanozoon* species and *T. congolense* rather than *T. vivax* which fail to grow in laboratory animals. Therefore, sub-inoculation using domestic small ruminants (sheep and goat) may be recommended for the isolation of *T. vivax* (Eisler *et al*., 2004; Molyneux, 1975).

A field study was conducted in Lambwe Valley area, Kenya, to evaluate the currently available trypanosome diagnostic techniques in domestic animals (Robson and Ashkar, 1972a). The study revealed that among the infected animals; *T. brucei* s.l. infections were diagnosed using the mouse inoculation method rather than the blood examination techniques (94.8% and 5.2%, respectively). However, more *T. congolense* were diagnosed using the blood examination methods than with the mouse inoculation method (87.5% and 43.3%, respectively). Moreover, *T. vivax* was only diagnosed by blood examination (20.2%) rather than by mouse inoculation (Robson and Ashkar, 1972a). The drawbacks of the sub-inoculation technique are mainly the time taken for trypanosomes to show in the inoculated laboratory animals, the need for groups at least six animals which makes this technique expensive and ethical considerations (Molyneux, 1975).

Xenodiagnosis

The other sub-inoculation method is xenodiagnosis which is feeding of a clean susceptible *Glossina* species on a suspected case of trypanosomiasis. Afterwards the fly is either dissected and examined for the presence of trypanosomes or allowed to feed on a clean animal which in turn is examined for the presence of trypanosomes. Although this method is found to be sensitive, it is rarely used due to the scarcity of laboratory reared *Glossina* species. This method is also time consuming and not likely to be a practical field technique (Eisler *et al*., 2004; Frezil, 1971; Molyneux, 1975).

In vitro isolation

A culture medium (Cunningham's medium) based on the amino-acid composition of tsetse hemolymph and containing 20% (v/v) foetal bovine serum was designed for the cultivation of various trypanosomatida (Cunningham, 1977). It was reported that Cunningham's media does not support the transformation of bloodstream forms from rodent blood to the procyclic phase (Bienen *et al*., 1980). However, the transformation was achieved using the natural cycle in the vector by feeding infected

blood to susceptible laboratory reared tsetse flies (Dukes *et al*., 1989). These findings were then investigated by Bass and Wang (1992) who showed that *T. brucei* s.l. was found to differentiate directly from the long slender bloodstream form to the procyclic form in Cunningham's medium at 26ºC without going through either the intermediary or the short-stumpy stage.

The development of a kit for the *in vitro* isolation of trypanosomes (KIVI) that allowed direct introduction of blood into culture medium, with the subsequent transformation and multiplication of procyclic trypanosomes solved this problem (Aerts *et al*., 1992). These kits were developed for the isolation of *T. b. gambiense* with a remarkable efficiency when compared with the rodent isolation procedure (Truc *et al*., 1992).

McNamara *et al*. (1995a) evaluated the in field use of KIVI for the isolation of *T. b. gambiense* in a sleeping sickness focus in northern Uganda. Blood samples positive for *T. b. gambiense* using serological tests were used for the isolation of the parasite using the kits. Only 80% of the isolates were able to grow in the culture kits. The authors explained the reported KIVI failures (20%) with the existence of certain *T. b. gambiense* strains that were unable to become established in the culture medium. Moreover, this could be also due to the insufficient stumpy forms present in the inoculum (McNamara *et al*., 1995a). Although the reported failure percentage of the kits, the authors recommended the use of KIVI for the isolation of the parasites as it is undoubtedly the best method available.

Field application of KIVI was reported also by Truc *et al*. (1997) who examined 122 wild mammals in Cote d'Ivoir for the identification of *T. b. gambiense*. The results revealed that 88 animals (72.1%) were positive using KIVI kits confirming the potential value of these kits as a diagnostic test for trypanosome infection. Moreover, out of 74 patients in a sleeping sickness focus of Cote d'Ivoir, 43 *T. b. gambiense* were isolated using KIVI kits (Jamonneau *et al*., 2004).

Although these kits were designed for the isolation of *T. b. gambiense*, they have been proven useful for detecting *T. brucei* and *T. congolense* in man or animals (McNamara *et al*., 1995b; Truc *et al*., 1992). Moreover, a study conducted by Verloo *et al*. (2000) suggested that KIVI is an excellent tool for isolation of *T. theileri* from cattle in Belgium. However, the easy growth of *T. theileri* might render KIVI less efficacious for the isolation of pathogenic trypanosomes (Verloo *et al*., 2000). This method is not practical for in field diagnosis of bovine trypanosomiasis due to the long pre-patent period (two to four weeks) before the culture becomes positive by microscopy and they are relatively expensive (Aerts *et al*., 1992; Eisler *et al*., 2004; Truc, 1996).

2.2.4 Immunodiagnostic techniques

Direct demonstration of trypanosomes in the infected animal or patient gives conclusive proof of infection. However, the limitations of parasitological diagnosis in terms of low sensitivity have been

the driving force for a great deal of research into alternative techniques that provide an indirect evidence of infection, namely immunodiagnostic techniques (Luckins, 1992).

2.2.4.1 Complement fixation test

Complenet fixation test (CFT) was used successfully in the control and eradication of dourine disease (caused by *T. equiperdium* in horses) in North America and surra disease (caused by *T. evansi* in buffaloes) in the Philippines, in which trypanosomes are rarely detected in blood or body fluids (Eisler *et al*., 2004; Luckins, 1992). This assay has not been found useful for the diagnosis of other trypanosome infections due to problems in reagent preparations, standardisation and anti-complementary activity in sera from several animal species (Luckins, 1992).

2.2.4.2 Indirect fluorescent antibody test

Indirect fluorescent antibody test (IFAT) has been used extensively in the detection of trypanosomal antibodies in animals and humans (Luckins, 1992). This test has been proven to be specific and sensitive for detecting trypanosome antibodies in infected cattle (Luckins and Mehlitz, 1978) and camels (Luckins *et al*., 1979). The major drawback of the IFAT is the occurrence of cross-reactions between different trypanosome species. This was clearly noticed from a study conducted by Ashkar and Ochilo (1972) who applied IFAT test for trypanosomiasis diagnosis in samples collected from cattle in the Lambwe Valley, Kenya. They found that more than 85% of examined cattle infected with *T. vivax* or *T. congolense* reacted with *T. brucei* s.l. antigen (Ashkar and Ochilo, 1972). Additionally, the requirement of sophisticated microscopy for the detection of fluorescence is considered an obstacle for using IFAT for field application (Luckins, 1992).

2.2.4.3 Card agglutination trypanosomiasis test

The card agglutination trypanosomiasis test (CATT) has been widely used for in field diagnosis of infections with *T. b. gambiense* because it is considered a rapid, cheap and practical serological test (Chappuis *et al*., 2004; Magnus *et al*., 1978). The antigens originate from particular variable antigen types (VATs) of *T. b. gambiense* that are highly conserved across the range of this species and they are expressed early with infection. Therefore, the majority of infected individuals develop antibodies that cause visible agglutination when whole blood or serum is mixed with the antigen on the card (Eisler *et al*., 2004; Luckins, 1992; Magnus *et al*., 1978). The sensitivity of the test for *T. b. gambiense* varies between 87% and 98%, with an average of 95% (Robays *et al*., 2004).

The test was used by Inojosa *et al*. (2006) for diagnosing human African trypanosomiasis in Angola. The authors screened 14,446 people using CATT; the results revealed that 1.7% reacted positive for the test. Although this low prevalence, the authors concluded that CATT is useful for initial screening but because the drug used for treatment is toxic, CATT should not be used alone for the treatment

decision (Inojosa *et al*., 2006). This test has also been used for the diagnosis of *T. evansi* (Njiru *et al*., 2004a) but unlikely to be of use for *T. vivax* or *T. congolense* due to the difficulty of identifying suitable variable antigens of these species (Luckins, 1992).

2.2.4.4 Enzyme linked immunosorbent assay

Enzyme linked immunosorbant assay (ELISA) has been used as a diagnostic test for trypanosomiasis caused by the different trypanosome species (Luckins, 1977, , 1992; Luckins *et al*., 1979; Luckins and Mehlitz, 1978). The use of ELISA for field diagnosis was based on the fact that the test may be performed without specialised equipment making it adaptable to inexpensive versions for use in the field or in under-resourced laboratories (Eisler *et al*., 2004; Luckins, 1992).

It was shown that Ab-ELISA is able to detect specific antibodies in trypanosome infected cattle with more detection of serologically positive cattle than the IFAT. However, similarly to IFAT, cross reactivity between the three major trypanosomes causing trypanosomiasis (*T. brucei* s.l., *T. congolense* and *T. vivax*) also occurred in Ab-ELISA (Eisler *et al*., 2004; Luckins, 1992; Luckins and Mehlitz, 1978). Evaluation of Ab-ELISA was done using antigen preparations for the diagnosis of *T. evansi* infection in cattle, the results revealed that the reaction was specific for this species although the sensitivity was low (Reid and Copeman, 2002).

Since the detection of antibodies does not necessarily indicate an active infection (because antibodies frequently persist far longer than the infectious agent); Ab-ELISA could not be used solely for diagnosis and instead another test is required at the same time. In cattle, antibodies were shown to persist inside the host for six to 13 months after the clearance of trypanosome infection (Eisler *et al*., 2004; Luckins, 1992; Van den Bossche *et al*., 2000). In the last decade, interest in the Ab-ELISA for bovine trypanosomiasis has focused on use as an epidemiological tool for mapping and quantifying trypanosomiasis prevalence and risk. In this context, Ab-ELISA will provide information on the aggregated level of trypanosome challenge over a prolonged period prior to the survey.

Alternatively, detection of the parasite itself rather than antibodies is important for identifying active infection; Ag-ELISA achieves this purpose by detecting the parasite antigen using the double sandwich ELISA method. Ag-ELISA was shown to detect *T. congolense* and *T. evansi* within 10-14 days of infection and these antigens disappeared from the animal within 21 days of trypanocidal drugs treatment (Rae and Luckins, 1984). Ag-ELISA was shown to have a high diagnostic sensitivity; more than 90% and 95% in cattle and camels, respectively (Nantulya, 1990).

For the ELISA to be used more widely, it will be necessary to provide training programmes providing a standardised assay protocol and reference preparations including suitable and defined control sera (Luckins, 1992).

2.2.5 Molecular diagnosis

The detection and identification of trypanosomes by molecular means should be based upon genetic markers that are able to reveal the presence of the parasite irrespective of the developmental stage of the parasite at the time of identification (Moser *et al.*, 1989a).

The genetic material of the trypanosome (Figure 2.12) is composed of nuclear DNA (nDNA) that is organised in chromosomes and kinetplastid DNA (kDNA). The nDNA content of *T. brucei* s.l. is about 35 Mb that probably harbours about 12,000 genes (El-Sayed *et al.*, 2000). The chromosomal DNA is grouped in three classes according to size. These groups are the megabase chromosomes (one to six Mb), intermediate chromosomes (200-900 kb) and the mini-chromosomes (50-150 kb). The kDNA represents about 20% of the total DNA and is organised in mini and maxi-circles. The mini-circles are 1 kb in size and found in 5000-20,000 copies, while, the maxi-circles are about 20 kb in size and found in 20-50 copies (Desquesnes and Davila, 2002; El-Sayed *et al.*, 2000). The nuclear and kDNA content of *T. brucei* s.l. was determined to be 0.097 and 0.004 pg, respectively (Borst *et al.*, 1982).

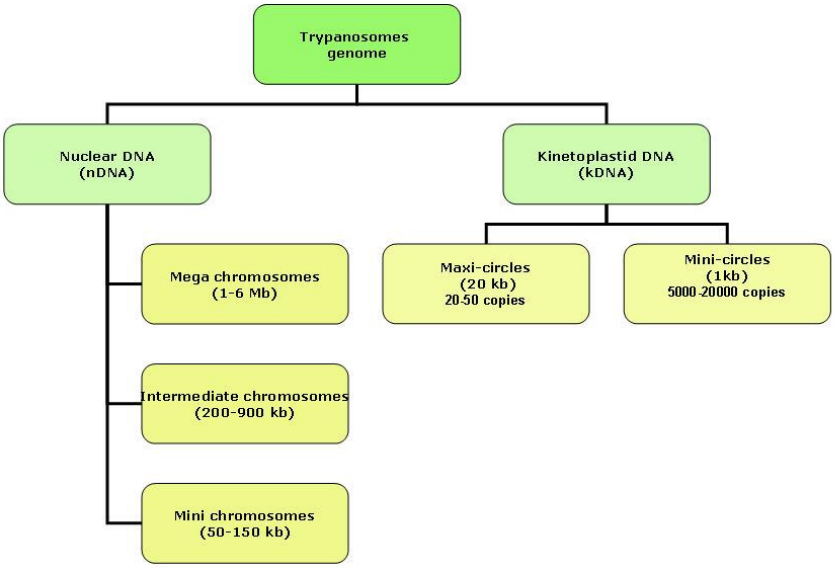


Figure 2.12: Composition of the African trypanosome genome (Desquesnes and Davila, 2002; El-Sayed *et al.*, 2000)

2.2.5.1 DNA hybridization

Identification of infectious agents using DNA probes has become a technique readily available for field epidemiologists and control organisers (ole-MoiYoi, 1987). The application of DNA probe technology has been a major breakthrough in the molecular diagnosis of trypanosomes, although it has revealed greater complexity than previously thought due to the discovery of new species and sub-species (Gibson, 2002). The important criteria for an identification method are sensitivity and specificity, realised in DNA probes based on species-specific repetitive DNA satellite sequences (Gibson, 2002; Sloof *et al*., 1983a). The satellite DNA is so-called because it bands separately from the bulk of the nuclear DNA in density centrifugation due to its high Adenosine+ Thymine (A+T) content (Gibson *et al*., 1988).

T. brucei s.l. complex

As mentioned above, in relation to the trypanosome genome, the *T. brucei* s.l. mini-chromosomes are linear mini-chromosomal DNA molecules of 50-150 kb. They are comprised predominantly of an internal tandem array of a 177 bp repeat (satellite DNA), which constitutes >90% of the sequence in some mini-chromosomes, over 10,000 copies of this repeated sequence were found to be present per trypanosome genome (El-Sayed *et al*., 2000; Sloof *et al*., 1983a; Sloof *et al*., 1983b). The analysis of satellite DNA from *T. brucei* s.l. revealed that the Guanidine+Cytocine (G+C) content is 29% and 71% A+T content (Sloof *et al*., 1983a). A *T. brucei* probe prepared by Gibson *et al*. (1988) based on the 177 bp repetitive sequence was tested against DNA from a range of trypanosome stocks. However, it hybridized to every *Trypanozoon* stock tested including *T. b. gambiense* and *T. evansi* due to the close similarity of species and sub-species within this sub-genera (Gibson *et al*., 1988).

T. congolense species

Enzyme polymorphism was used extensively to investigate sub-specific diversity and the genetics of a wide variety of organisms, such methods revealed a distinct enzyme polymorphism in *T. congolense* (Young and Godfrey, 1983). Two stocks of *T. congolense* isolated from a sheep and a goat on the Kenya coast were found to be distinctly different from 112 other stocks of the same species after comparing by isoenzyme electrophoresis. This difference indicated a marked genetic distinction that may be of epidemiological significance (Gashumba, 1986). Knowles *et al*. (1988) reported the elecrophoretic variation of enzymes found in stocks of *T. congolense* taken from two areas on the Kilifi area of the Kenya coast. Moreover, the DNA from the Kilifi parasites contained a repetitive DNA sequence that was absent from other stocks of *T. congolense*.

The main groupings of *T. congolense* based on isoenzyme electrophoresis were reported by Gashumba *et al*. (1988) who summarised these groups to three, Savannah group (has two subgroups: East African Savannah and East/West African Savannah), Riverine/Forest group and the Kenya coast group.

Furthermore, differences in the molecular karyotypes, repetitive DNA sequences and the kDNA sequences suggested that there are some trypanosome populations that are classified as *T. congolense* based on the morphological features, but differ significantly from each other at the molecular level (Majiwa *et al*., 1986; Majiwa *et al*., 1985).

In order to determine and compare the pathological effects induced by the three types, Majiwa *et al*. (1985) and Bengaly *et al*. (2002) experimentally infected three groups of Zebu cattle with the different species. The study revealed that *T. congolense* Savannah was of high pathogenicity that led to progress of the disease and ended by death within four to seven days post infection. The low pathogenicity of Forest type was observed with a self-cure by clearing the infection that suggested the ability of cattle to eliminate this trypanosome type. Moreover, the Kilifi type was found to be asymptomatic suggesting the ability of the host to control this type of infection.

The mini-chromosomes of *T. congolense* Savannah are characterised by being very small in size, that they are 25-50 kb (Gibson and Borst, 1986). They contain many repeats of a satellite DNA sequence of 369 bp that constitutes an estimated 5% of the nDNA. This implies that the repetitive sequences are present at about 5400 copies in a single parasite if the genome size is $4x10^7$ (Gibson *et al.*, 1987; Katakura *et al.*, 1997; Masiga *et al.*, 1992; Moser *et al.*, 1989a). The DNA sequence of the satellite DNA from *T. congolense* Savannah was found to have 65% A+T content (Masiga *et al*., 1992). DNA probes specific for *T. congolense* Savannah, *T. congolense* Forest and *T. congolense* Kilifi targeting 369 bp, 350 bp and 400 bp fragments, respectively, were described by Gibson *et al*. (1988). These probes were specific for the corresponding species and there was no cross hybridization with trypanosomes of other species (Gibson *et al*., 1988).

T. vivax species

A *T. vivax* DNA probe was prepared by Kukla *et al*. (1987) using purified *T. vivax* DNA. The purified DNA was digested with the restriction enzyme *EcoRI* and the fragments were then cloned at the *EcoRI* site of the bacteriophage lambda gtWES. The recombinant phages were propagated in *E. coli* strain and transferred on to nitrocellulose filters and hybridised to radio-isotope labelled homologous total *T. vivax* DNA. This probe was used to hybridise with *T. vivax* infected flies by examining the proboscis of the flies. The target for such probe is the satellite DNA sequence which does not exist in all *T. vivax* isolates. Therefore, Masake *et al*. (1994) designed another probe targeting a fragment of the gene encoding *T. vivax* specific antigen which occurs in all *T. vivax* isolates examined by the authors.

T. simiae species

DNA probe targeting the 200 bp satellite sequence of *T. simiae* was described by Gibson *et al*. (1988) and showed hybridization to the specific species without cross reaction with other species.

Applications of DNA probes

Application of DNA probes for the identification of trypanosome infections in wild tsetse flies has been reported from the Gambia (McNamara *et al.*, 1989). The probes identified 17 flies out of 52 examined (33%) to be infected, the species were diagnosed to be *T. simiae* (11 out of 17) and six *T. congolense*. McNamara and Snow (1990) collected flies from the same area surveyed by McNamara *et al*. (1989) for the identification of trypanosome species using DNA probes. Twenty eight infections (78%) were identified using DNA probes specific for *Trypanozoon*, *T. simiae* and the three types of *T. congolense* (McNamara and Snow, 1990). In Cote d'Ivoir, McNamara *et al*. (1995b) examined midguts of 30 flies for the presence of trypanosomes using DNA probes specific for *Trypanozoon*, the three types of *T. congolense* and *T. simiae*. Probe hybridization detected only one single infection with *T. congolense* Forest, 10 double infections and 19 triple infections with the three types of *T. congolense* species.

2.2.5.2 PCR

In recent years, PCR has been developed and became widely used for the detection of trypanosomes. This approach has overcome the constraints of parasitological and serological techniques (Gonzales *et al.*, 2003; Moser *et al.*, 1989a). PCR has proven to be highly sensitive and specific for trypanosome detection (Becker *et al*., 2004). The use of PCR in detecting the trypanosome DNA is reported to be the most reliable and accurate technique to assess and followup the efficacy of trypanosomiasis treatment (Njiokou *et al*., 2004; Truc *et al*., 1999). In a study to compare the use of Ag-ELISA and PCR in detecting trypanosomes in experimentally infected flies, Ouma *et al*. (2000) showed that Ag-ELISA and PCR detected 71.4% and 96% of *T. congolense* and 98.4% and 97.6% of *T. brucei* s.l. infections, respectively, indicating a high sensitivity of detecting trypanosomes using PCR.

It was observed that PCR can detect the DNA equivalent of one trypanosome/10 ml of host (cattle) blood (Masake *et al*., 2002), while, in human blood, the detection limit of PCR is 25 trypanosomes/ml (Kanmogne *et al*., 1996). The presence of human DNA is an important factor that interferes with the reaction yield of the PCR; this was described by Contamin *et al*. (1995) who noticed a decrease of the PCR sensitivity in identifying malaria parasites in human blood by 10 fold due to the presence of human DNA compared to amplification of purified parasite DNA.

The coming section summarises the different types of PCR used for trypanosome species identification.

2.2.5.2.1 Species-specific PCR reactions

T. brucei s. l.

Many studies were conducted to design primers targeting DNA sequences or genes specific for *Trypanozoon* species identification. Table 2.5 summarises the different primers used with the size of the amplified product and the type of the target.

Table 2.5: *T. brucei* s.l. different primers for PCR

Reference	Target	Identified species	Size of amplified product	Sequence
(Moser *et al.*, 1989a)	177 bp repetitive satellite sequence	*Trypanozoon*	173 bp	TBR1:5'-CGA ATG AAT ATT AAA CAA TGC GCA GT-3'
				TBR2: 5'-AGA ACC ATT TAT TAG CTT TGT TGC-3'
(Masiga *et al*., 1992)	177 bp repetitive satellite sequence	*Trypanozoon*	164 bp	TBR1: 5'- GAA TAT TAA ACA ATG CGC AG-3'
				TBR2: 5'- CCA TTT ATT AGC TTT GTT GC-3'
(Becker *et al.*, 2004)	177 bp repetitive satellite sequence	*Trypanozoon*	134 bp	Tb177F: 5'- AAC AAT GCG CAG TTA ACG CTA T-3'
				Tb177R: 5'- ACA TTA AAC ACT AAA GAA CAG CGT TG-3'
(Majiwa *et al*., 1994)	5.2 kb repetitive ingi sequence	*Trypanozoon*	1500 bp	ILO342: 5'-GAT CCG CAG CCG GGC CTG-3'
				ILO343: 5'-CCG CGG TGG CTC CTT CCC-3'
(Kabiri *et al*., 1999)	ESAG 6 and 7	*T. brucei* s. l.	286 bp	Museq1: 5'-GCG TTA GCA GCA GCT GCA GCT GGG-3'
				Museq2: 5'-CCT CCT CGG ATA TTT TCC GCA CCC-3'
(de Almeida *et al.*, 1998a)	1.4 kb DNA sequence encoding mRNA spliced leader	*Trypanozoon*	246 bp	ORPHON5J-u: 5'- GAT CCC TCT CCA CCA ATC CG-3'
				ORPHON5J-1: 5'- AAC TGC CCC GAC CTC CGC AGT-3'
(Artama *et al*., 1992)	Procyclin gene repeat	*Trypanozoon*	300-400 bp	5'-CAC AAT GGC ACC TCG TTC CC-3'
				5'- TTA GAA TGC GGC AAC GAG A-3'
(Artama *et al*., 1992)	1.35 kb DNA sequence encoding mRNA spliced leader	*Trypanozoon*	1350 bp	5'- TAG CGT TAG TTG AAA GC-3'
				5'- TAT TAT TAG AAC AGT TTC TGT AC-3'
(Picozzi *et al*., 2008)	PLC gene	*Trypanozoon*	324 bp	PLC1: 5'- CGC TTT GTT GAG GAG CTG CAA GCA-3'
				PLC2: 5'- TGC CAC CGC AAA GTC GTT ATT TCG-3'

As mentioned before, a satellite DNA sequence of 177 bp length specific for *Trypanozoon* was described by Sloof *et al*. (1983a) and used as a target for the identification of this genus by PCR. This target was the most favourable targets due to the high copy number (10,000) thus increasing the chance that the trypanosome DNA would be amplified (Desquesnes and Davila, 2002). Moser *et al*. (1989a) reported that TBR universal primers that are used to prime the amplification of 177 bp sequences from *Trypanozoon* genomic DNA, are able to detect 0.1 pg of parasite DNA, this is about the amount of DNA calculated to be in a single trypanosome. Moreover, weak bands containing the

amplification product could be detected when the DNA content was 0.01 pg which is equivalent to $1/10^{th}$ that of a single parasite. This is in agreement with Clausen *et al*. (1998) who found that *T. brucei* s.l. specific nuclear repeat primers amplified 177 bp satellite sequence are able to detect as little as one femtogram purified DNA which is equivalent to about 1% of the genome content of one parasite.

Different primers targeting this satellite sequence were designed to target specific regions of the target. For instance, primers amplifying 173 bp product of the target were described by Moser *et al*. (1989a) and other primers were published by Masiga *et al*. (1992) amplifying 164 bp of the same target sequence. Becker *et al*. (2004) re-designed the primers previously designed by Moser *et al*. (1989a) to get rid of the tendency of the primers to form secondary structures to be used in qPCR.

The primers targeting the repetitive 177 bp sequence are considered universal and have been used in different studies for the identification of *T. brucei* s.l. in animal hosts (Clausen *et al*., 1998; Gonzales *et al*., 2003; McOdimba, 2006; Picozzi *et al*., 2002) and vector (Boid *et al.*, 1999; Morlais *et al.*, 1998b; Morlais *et al.*, 1998a; Njiru *et al.*, 2004b).

Another sequence specific for *Trypanozoon* reported by Kimmel *et al*. (1987) was used as a target for PCR amplification. This sequence is a repetitive element of 5.2 kb named *ingi* which has the Kiswahili root adjective meaning many. This target is present in 200 copy numbers in the *Trypanozoon* genome and believed to be specific for *Trypanozoon* only as it has not been detected in isolates of other genera such as *T. congolense* and *T. vivax* (Kimmel *et al*., 1987). Primers targeting these sequences were designed by Majiwa *et al*. (1994) and used for detection of *Trypanozoon* infections in the saliva of tsetse flies and buffy coat samples from cattle. The designed primers amplified a product of 1500 bp length (Majiwa *et al*., 1994).

In *T. brucei* s.l. there are 20 copies of the expression site associated genes (ESAG) 6 and 7 in the trypanosome genome (Kabiri *et al*., 1999). These targets are utilised by PCR for the detection and identification of *T. brucei* s. l. The detection limit of PCR using these primers was reported to amplify a parasite DNA content equivalent to $1/10^{th}$ that of a single trypanosome in the reaction. This detection limit is equivalent to the detection of 40 trypanosomes/ml blood. These primers were used for the detection of *T. b. gambiense* in blood samples collected from patients suspected of having sleeping sickness in Equatorial Guinea and Angola (Kabiri *et al*., 1999).

The ORPHON5J primers targeting 246 bp fragment of 1.4 kb DNA sequence that encodes mRNAs termed spliced leader (Parsons *et al*., 1986) occurring in all *Trypanozoon* species were designed by de Almeida *et al*. (1998b). The target element was reported to be located in 100-200 copy number as tandemly repeated units in the genome (Parsons *et al*., 1986). The problem encountered with these primers was the cross-reactivity with *T. vivax*, whereas they do not react with *T. congolense*. The

authors argued that the more conventional *T. brucei* s.l. primers described by Moser *et al*. (1989) react with *T. vivax* and *T. congolense* stocks they examined (de Almeida *et al.*, 1998b).

Artama *et al*. (1992) described primers to amplify two different repetitive regions in the *T. brucei* s.l. nDNA. Firstly, primers targeting a tandem repeat of two similar adjacent procyclin genes were designed to amplify 300-400 bp products representing different procyclin genes. The procyclin gene target is reported to be found three times in the nDNA (Clayton *et al*., 1990). Surprisingly, PCR product was detected in *T. evansi* despite the fact that procyclin is not known to be required during the mechanically transmitted *T. evansi* infections (Artama *et al*., 1992). The second is the 1.35 kb repeat containing the spliced leader gene of which there are approximately 200-250 copies in the *Trypanozoon* nuclear DNA (Dorfman and Donelson, 1984).

The variant surface glycoprotein (VSG) of the mammalian bloodstream form of *T. brucei* was amongst the first proteins to be reported to be attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor linked to the C-terminal α-carboxyl group through ethanolamine (Carrington *et al*., 1989). Specific phospholipases that convert GPI-anchored proteins from membrane attached to soluble forms have been isolated from *T. brucei* s.l. (Hereld *et al*., 1986). Primers targeting the single copy PLC gene (Mensa-Wilmot *et al*., 1990) specific for *Trypanozoon* were reported by Picozzi *et al*. (2008) where they were used as an internal control using a multiplex PCR in combination with primers targeting the serum resistance associated gene (SRA) for the identification of *T. b. rhodesiense*.

T. b. rhodesiense

It is important to differentiate between *T. b. rhodesiense* and other *T. brucei* s.l sub species due to the inability to differentiate them morphologically. The aforementioned PCR reactions amplify different targets in *T. brucei* s.l. and *Trypanozoon* genomes but cannot differentiate between the different sub species. Human serum is lytic to *T. b. brucei,* however *T. b. rhodesiense* and *T. b. gambiense* are resistant to lysis by human serum enabling them to stablish and cause the diseases in this host.

De Greef *et al*. (1989) reported the presence of laboratory strains that were sensitive to lysis by human serum. The only molecular difference between the resistant and sensitive forms was a 1.5 kb mRNA, cDNA screening identified the gene, termed the serum resistant associated gene (SRA) in the resistant strains (De Greef *et al*., 1989; Xong *et al*., 1998). This gene alone was sufficient to confer human serum resistance on *T. b. brucei* by transfection (Xong *et al*., 1998). The resistance of *T. b. rhodesiense* to the lytic factor is determined by the N-terminal α-helix of SRA which is involved in coil-coiling with the trypanolytic factor by strong and specific binding to the C-terminal α-helix of apoL-I (Pays, 2006).

SRA has been shown to be a robust and ubiquitous marker for *T. b. rhodesiense* to distinguish this isolate from *T. b. brucei* and other trypanosomes of subgenus *Trypanozoon* (Gibson, 2002; Welburn *et al.*, 2001). Different primers have been designed for the amplification of SRA gene (Table 2.6).

Table 2.6: Different primers used to prime the amplification of SRA gene for *T. b. rhodesiense* identification

Reference	Size of amplified product	Primer sequence
(Welburn *et al*., 2001)	SRA: 743 bp VSG: 1121 bp	B537: 5'- CCA TGG CCT TTG ACG AAG AGC CCG-3'
		B538: 5'-CTC GAG TTT GCT TTT CTG TAT TTT TCC C-3'
(Gibson, 2002)	1.2 kb	SRA A: 5'-GAC AAC AAG TAC CTT GGC GC-3'
		SRA B: 5'-CAG CAA CCA TAT TCA GAG CC-3'
(Radwanska *et al*., 2002a)	284 bp	F: 5'-ATA GTG ACA AGA TGC GTA CTC AA CGC-3'
		R: 5'-AAT GTG TTC GAG TAC TTC GGT CAC GCT-3'
(Picozzi *et al*., 2008)	PLC: 239 bp SRA: 669 bp VSG: > 1 kb	02: 5'- GGA GCC AAA ACC AGT GGG CAC ATC-3'
		03: 5'- AAG TAG CGC TGT CCT GTA GAC GCT TC-3'
		651: 5'- GAA GAG CCC GTC AAG AAG GTT TG-3'
		652: 5'- TTT TGA GCC TTC CAC AAG CTT GGG-3'

Welburn *et al*. (2001) designed primers that amplified 743 bp sequences in the SRA gene to validate the use of the single copy SRA gene as a diagnostic indicator of *T. b. rhodesiense*. SRA gene is very similar to that of the VSG, but has an internal deletion of 378 bp that encodes the central part of variable surface glycoprotein N-terminal domain. These primers were designed to amplify across this deletion to distinguish the SRA product from any SRA-related variable surface glycoprotein (1121 bp) (Welburn *et al*., 2001). The authors identified the SRA gene in 29 samples collected from patients with sleeping sickness in Tororo district, Uganda. Moreover, of 200 cattle sampled in Soroti district, 18% were found infected with *T. b. rhodesiense*.

Radwanska *et al*. (2002a) evaluated the SRA gene as a tool for identification of *T. b. rhodesiense* strains using PCR. Twenty five previously identified *T. b. rhodesiense* isolates using the human serum resistance test were assessed using SRA-PCR. Twenty four isolates were identified as *T. b. rhodesiense* using SRA primers that prime the amplification of 284 bp sequence of the SRA gene. However, one strain was negative and was thought to be misidentification of the strain (Radwanska *et al.*, 2002a). The primers designed by Gibson *et al*. (2002) to amplify 1.2 kb sequence of SRA gene were evaluated for the identification of *T. b. rhodesiense* from 264 trypanosome isolates from human, different animals and tsetse flies collected from 1964-2001 from sleeping sickness foci in East Africa (Njiru *et al.*, 2004c). In total, 75 *T. brucei* s.l. isolates were found to have SRA gene confirming that SRA gene is a specific marker for *T. b. rhodeisense* hence offering an opportunity to detect the animal host and targeting them using different trypanocidal drugs.

In another study, the primers designed by Radwanska *et al*. (2002) and Gibson *et al*. (2002) were evaluated for the amplification of SRA in samples collected during a cross sectional study in Uganda (Enyaru *et al*., 2006). SRA was identified in 28 (80%) of the 35 *T. b. rhodesiense* isolates from

parasitologically confirmed sleeping sickness cases, using primers designed by Radwanska *et al*. (2002). While, the gene was identified in 27 (77.1%) of the same 35 *T. b. rhodesiense* isolates using the primers designed by Gibson *et al*. (2002).

The fact that SRA is a single copy gene makes the interpretation of a negative result difficult, as in such cases, negative result is either due to absence of SRA gene or the low level of parasitaemia (Katunguka-Rwakishaya, 1996). To overcome this problem, a multiplex PCR was developed by Picozzi *et al*. (2008). The reaction contains two different sets of primers to amplify two products within the same reaction from the same starting material. The primers target both SRA gene for the diagnosis of *T. b. rhodesiense* and GPI-PLC which is found in *T. brucei* s.l. genome as an internal control. Positive amplification of GPI-PLC indicates sufficient *T. brucei* s.l. genomic material is present to detect a single copy gene and the presence or absence of SRA determines whether *T. b. rhodesiense* is present (Picozzi *et al*., 2008). The amplification of GPI-PLC only indicates that the sample is negative for *T. b. rhodesiense*, whereas, it might be positive for *T. b. brucei*, *T. b. gambiense* or *T. evansi*. However, concerning both the host range and geographic distribution of these parasites, the assumption that the absence of the SRA amplicon indicates the presence of *T. b. brucei* is made.

T. b. gambiense

T. b. gambiense is so closely related to the rest of the *T. brucei* s.l. complex, therefore, no *T. b. gambiense*-specific DNA probe has been reported (Bromidge *et al*., 1993). However, certain VSG genes, for example LiTat 1.3, the antigen used in the serological card agglutination test, was used for identification of *T. b. gambiense* (Magnus *et al*., 1978). In Gambian sleeping sickness foci, PCR reactions targeting *Trypanozoon* subgenus markers (Table 2.7) were used, however, these results were not satisfactory due to inability to distinguish between *T. brucei* s.l. species (Kabiri *et al*., 1999; Kanmogne *et al*., 1996; Penchenier *et al*., 2000).

Table 2.7: Primers designed for PCR identification of *T. b. gambiense*

Reference	Target	Size of amplified product	Primer sequence
(Bromidge *et al*., 1993)	AnTat 11.17 VSG gene	653 bp	AnTA: 5'-CAC AGA CGA CAG AAG CGA TA-3'
			AnTB: 5'-GAA AGT GGG AGT TGT TGC TC-3'
(Mathieu-Daude *et al*., 1994)	Non conserved sequence of kDNA	930 bp	MDF: 5'- CGC CCA TAA GAT TTC CGG TT-3'
			MDR: 5'- GGT GTA ATA CTC ACC CGG TT-3'
(Schares and Mehlitz, 1996)	Non conserved sequence of kDNA	930 bp	MDF: 5'- CGC CCA TAA GAT TTC CGG TT-3'
			MDR: 5'- GGT GTA ATA CTC ACC CGG TT-3'
			MDN: 5'-CGA GGT ACT TCG AAA GGG TT-3'
(Radwanska *et al*., 2002b)	TgsGP	308 bp	Sense primers: 5'- GCT GCT GTG TTC GGA GAG C-3'
			Anti-sense primers: 5'- GCC ATC GTG CTT GCC GCT C-3'

PCR reaction was developed by Bromidge *et al*. (1993) to detect the AnTat 11.17 VSG gene which is capable of distinguishing *T. b. gambiense* from other *T. brucei* s.l. group trypanosomes in most foci of Gambian sleeping sickness. However, this target gene was absent in six stocks collected from the Moyo focus in West Uganda meaning the difficulty of using VSG genes as target markers for the identification due to instability of these genes.

As shown previously in Figure 2.12, the one kilobase minicircles (10,000 copies) contain 177 bp sequences that appear to be conserved within *T. brucei* s.l. These minicircles contain also non-conserved sequence (hypervariable region, HVR) that is heterogenous within a single organism (200-300 different sequence classes of HVR). They were used to design specific probes for *T. cruzi* (Veas *et al*., 1990), therefore, HVR were used in the same manner to design specific markers for *T. b. gambiense* using PCR (Mathieu-Daude *et al*., 1994). However, this technique has been used only with large parasite number (103 trypanosomes per reaction) obtained after *in vitro* cultivation of procyclic trypanosomes and was not evaluated for use with in field blood samples. Therefore, Schares and Mehlitz (1996) used nested PCR for the amplification of kDNA non-conserved sequence from blood samples isolated from animals and patients in sleeping sickness foci in Zaire. The primers used were the forward and reverse ones designed by Mathieu-Daude *et al*. (1994) and another primer set for the nested reaction. The results showed that one parasite per reaction volume was able to produce a strong amplicon in the nested PCR and the samples collected from the field were successfully identified as *T. b. gambiense* infected using the nested reaction.

The sequence of the *T. b. gambiense* specific glycoprotein (TgsGP) which encodes a flagellar pocket glycoprotein, were found to be diagnostic for *T. b. gambiense* only (Berberof *et al*., 2001). Primers

targeting this sequence were designed by Radwanska *et al.* (2002b) and were found to be specific for the amplification of TgsGP of *T. b. gambiense* when tested against non-*T. b. gambiense* populations revealing negative results. The detection limit of the reaction was evaluated; it reached 1000 trypanosomes/ml of blood after a single PCR. This detection limit was increased to 10 trypanosomes/ml of blood when the TgsGP PCR was repeated using aliquot of the first PCR product. The diagnostic potential of the TgsGP-PCR was evaluated on a collection of human blood samples from Cote d'Ivoire and Belgium. Of 41 CATT-positive samples from Cote d'Ivoire, 14 were positive and they were confirmed using parasitological methods.

T. congolense

The satellite sequence specific for the three sub species of *T. congolense* have been used to identifiy these trypanosomes using PCR. The primers targeting the satellite fragments of *T. congolense* Savannah, *T. congolense* Forest and *T. congolense* Kilifi are summarised in Table 2.8.

Table 2.8: Primer sequences used to target satellite DNA marker of *T. congolense*

Reference	Target	Size of amplified product	Primer sequence
(Masiga *et al.*, 1992; Moser *et al.*, 1989)	Satellite DNA of *T. congolense* Savannah	316 bp	TCS1: 5'-CGA GAA CGG GCA CTT TGC GA-3'
			TCS2: 5'-GGA CAA ACA AAT CCC GCA CA-3'
(Masiga *et al.*, 1992)	Satellite DNA of *T. congolense* Forest	350 bp	TCF1: 5'- GGA CAC GCC AGA AGG TAC TT-3'
			TCF2: GTT CTC GCA CCA AAT CCA AC-3'
(Majiwa *et al.*, 1994)	Satelite DNA of *T. congolense* Kilifi	400 bp	TCK1: 5'-GCT GCA GGT CGA CGG ATC-3'
			TCK2: 5'- CCC TCG AGA ACG AGC A-3'

The Savannah group is the most widespread, in terms of both geographical and host range, overlapping with the Forest and Kenya Coast sub-species where they occur in the humid parts of West and Central Africa and Kenya, respectively (Gibson *et al.*, 1988; Masiga *et al.*, 1996; Reifenberg *et al.*, 1997b; Young and Godfrey, 1983).

The sensitivity of the universal primers in detecting *T. congolense* Savannah was found to be 0.1 pg of parasite DNA which is about the amount of DNA calculated to be in a single trypanosome. Moreover, weak bands containing the amplification product could be detected when the DNA content was 0.01 pg which is equivalent to $1/10^{th}$ that of a single parasite (Moser *et al.*, 1989a). Although the similarity in the sequence of satellite DNA of *T. congolense* Savannah compared with *T. brucei* s.l. and *T. vivax* (44 and 37%, respectively), this doesn't affect the specificity of the primers to detect the target sequence (Masiga *et al.*, 1992). This was studied by Gibson *et al.* (1987) who introduced the mini-chromosomes from *T. congolense* Savannah into *T. brucei* s.l. using electroporation. They were able

to detect both satellite sequences indicating that these sequences are ideal for use to distinguish trypanosome species and subspecies.

In field, the primers specific for the three *T. congolense* sub species were used to identify infections in tsetse flies in Cameroon (Morlais *et al.*, 1998b; Morlais *et al.*, 1998a). PCR for detecting *T. congolense* Savannah and *T. congolense* Kilifi were used on samples collected from saliva of tsetse flies and buffy coat from aparasitaemic cattle (Majiwa *et al.*, 1994). Also, these primers were used to detect the *T. congolense* sub species in blood samples collected from cattle in Uganda (Clausen *et al.*, 1998; McOdimba, 2006). Njiru *et al.* (2004b) identified the three sub species of *T. congolense* using PCR in 3826 *Glossina* species collected from Kenya. The different primers identified 1.2% infections with *T. congolense* Savannah, 0.7% *T. congolense* Kilifi and 0.6% *T. congolense* Forest infections. Moreover, PCR was able to identify mixed infections with different *T. congolense* sub species which is not possible using microscopy.

T. vivax

The natural home for *T. vivax* is tropical Africa where it is prevalent in most parts of West, Central, East and South Africa (Hoare, 1972). This species represents a remarkable instance of a tsetse-borne trypanosome that has spread far beyond its original area of distribution to distant areas such as West Indies, Central and South America through imported cattle from Africa (Hoare, 1972). In addition to tsetse flies, which are the true vectorial hosts of *T. vivax*, it is readily transmitted by mechanical inoculators represented by other bloodsucking Dipetra, especially Tabanid flies (Desquesnes and Dia, 2003; Desquesnes and Dia, 2004; Hoare, 1972).

The diagnosis of *T. vivax* based on the presence of specific insect vectors is limited due to the ease of transmission by a variety of insect vectors in addition to the tsetse fly. Diagnosis using clinical picture of the disease and using microscopy is also limited due to the absence of a consistent set of clinical signs (hemorrhagic form in Kenya cost and sub clinical infection in West Africa) and the often very low parasitemia of infections (Masake *et al*., 1997). Infection of cattle with certain stocks of East African *T. vivax* has been associated with varying degrees of an intravascular coagulation-like syndrome which is occasionally accompanied by severe haemorrhage (Masake *et al*., 1994). The American isolates induce a disease generally more severe than those from Africa (Gardiner, 1989).

T. vivax has a similar molecular karyotype to other salivarian trypanosomes, with chromosomal DNA ranging in size from approximately 50-6000 kb. However, unlike the other salivarian trypanosomes which have an estimated 100 mini-chromosomes of 50-150 kb, *T. vivax* has only one or two mini-chromosomes which are characterised by the presence of a highly repetitive G+C rich (64%) satellite DNA of 180 bp in size (Dickin and Gibson, 1989; Stevens and Brisse, 2004). The kDNA mini-circles of *T. vivax* are approximately half the size of the other salivarian trypanosomes, while, the relative amount of maxi-circle DNA is at least twice that of *T. brucei* s.l.

Diagnosis of *T. vivax* infection using molecular markers has been subjected to a number of researches (Desquesnes, 1997; Masake *et al.*, 1997; Masake *et al.*, 1994; Masiga *et al.*, 1992; Morlais *et al.*, 1998b; Morlais *et al.*, 1998a; Morlais *et al.*, 2001). The satellite DNA sequence of *T. vivax* that is positioned in the mini-chromosomes has been used to identify infections with *T. vivax* (Dickin and Gibson, 1989; Masiga *et al*., 1992). However, it has been shown that this DNA sequence is not present in some *T. vivax* isolates; consequently, universal primers targeting a fragment of the gene encoding *T. vivax* specific antigen have been described by Masake *et al.* (1994).

The antigen is recognised by a monoclonal antibody (Tv27) in an Ag-ELISA, the cloned gene was found to be highly tandemly repeated with a length of 900 bp in the genome of all *T. vivax* isolates from diverse geographic locations in Africa and South America (Masake *et al*., 1994). Several primer pairs were tested for their capacity to amplify a segment of the repeat unit of the gene fragment (1870 bp long) but only one was able to amplify a segment of DNA from all the *T. vivax* tested isolates. The primer set was named ILO1264 and ILO1265 that amplified a segment of 400 bp (Masake *et al*., 1997). Table 2.9 summarises the sequences of the primers specific for *T. vivax* amplification.

Table 2.9: Primer sequences and amplified target sizes specific for *T. vivax*

Reference	Target	Size of amplified product	Primer sequence
(Masake *et al.*, 1994; Masiga *et al.*, 1992)	Satellite DNA	175 bp	TVW1: 5'-GTG CTC CAT GTG CCA CGT TG-3'
			TVW2: 5'-CAT ATG GTC TGG GAG CGG GT-3'
(Masake *et al.*, 1997)	Gene encoding specific antigen	400 bp	ILO1264: 5'- CAG CTC GGC GAA GGC CAC TTC GCT GGG GTG-3'
			ILO1265: 5'- TCG CTA CCA CAG TCG CAA TCG TCG TCT CAA GG-3'

A study comparing the sensitivity of primers targeting the 400 bp fragment of the species-specific antigen (ILO) and those targeting the satellite DNA (TVW) in the identification of *T. vivax* was conducted by Morlais *et al.* (2001). The ILO and TVW primers were used to identify *T. vivax* in 36 tsetse with mouthpart infections. The results revealed that ILO primer set identified *T. vivax* infections in 31 of the 36 mouthpart infections (86%), while TVW primers wrongly classified 16 (44%) of these infections as negative, indicating better sensitivity of ILO primers. These results demonstrate that the DNA sequence targeted by TVW primer set is not conserved in all *T. vivax* isolates, concluding that ILO primers are universal primers suitable for *T. vivax* diagnosis in hosts and vectors from different geographic areas. Moreover, in a study to compare the ability of ILO and TVW primers to detect the target sequences using *T. vivax* strains from West Africa, Lefrancois *et al.* (1998) clarified that primers used for amplifying diagnostic antigen sequence specific for *T. vivax* (ILO) are more specific and universal than the satellite sequence primers (TVW) that were unable to amplify DNA of all *Duttonella* stocks circulating in West Africa.

This is in agreement with the results obtained by Morlais *et al*. (1998a) who used TVW primers to amplify *T. vivax* strains infecting tsetse flies examined in Cameroon. The primers could not detect the parasite and the authors contributed this to the fact that these primers are designed from sequenced isolates of West African origin, and they may not anneal if different subtypes of *T. vivax* occur in Cameroon. Additionally, Malele *et al*. (2003) found that the use of the universal *T. vivax* primers rather than the primer set designed for the West African isolates doubled their detection of the parasite from tsetse flies collected in Tanzania. In Kenya, the detection of *T. vivax* using the universal primers improved the results obtained by Njiru *et al*. (2004b) who screened 3826 tsetse flies for trypanosome infections. The detection rate was improved from 0.2% to 0.7% indicating the occurrence of genetic variation of *T. vivax* in Kenya and the accurate detection using the universal primers compared to the West African isolates primers.

Conversely, Gonzales *et al*. (2003) reported that some stocks of *T. vivax* from Bolivia (South America) were unable to give a product using ILO primers suggesting that these strains do not have this sequence in their DNA, however, using TVW primers resulted in the detection of such stocks concluding the presence of the sequence targeted by TVW primers in the isolates occurring in Bolivia. This difference in the data from Bolivia in the sensitivity of the ILO primers could be due to variation in the processing of samples or technical problems in the procedure (Masake *et al*., 1997). In conclusion, by targeting a species-specific antigen, the ILO primer set showed better sensitivity and seems to provide a more reliable marker for *T. vivax* identification (Morlais *et al*., 2001).

2.2.5.2.2 Internal transcribed spacer-PCR as a pan-trypanosome reaction

In trypanosomes, as in other eukaryotic organisms, the ribosomal RNA (rRNA) genes are transcribed as a large primary transcript, which is processed by stepwise endonucleolytic cleavage to yield the 18S, 5.8S and 28S units (Urakawa and Majiwa, 2001). These RNAs interact with ribosomal proteins to form the ribosomes. Ribosomal RNA genes are highly conserved and have been proven to be useful in comparisons of more closely related species. Eukaryotic rRNA genes are found as tandem repeat of units separated by non-transcribed spacers (NTS) region and internal transcribed spacer regions (ITS). The ITS regions are relatively small, show variability among related species and are flanked by highly conserved segments to which PCR primers can be designed (Cupolillo *et al*., 1995). The inter-species length variation makes the ITS region a useful marker for species differentiation in trypanosomes (Cox *et al.*, 2005; Desquesnes *et al.*, 2001; McLaughlin *et al.*, 1996; Njiru *et al.*, 2005). At least, five PCR reactions using species-specific primers would be required for each sample depending on the host species and the geographic distribution of the trypanosome species compared to the convenience of a single test. Trials have been conducted to combine the species-specific primers in a multiplex reaction, but these trials have been shown to be less sensitive with some combinations of primers inducing the synthesis of non-specific and un expected size products (Desquesnes *et al*., 2001).

The most important obstacle of using species-specific primers is the lack of specific primers for all trypanosome species, leading to the inability of identifying new trypanosome species. For example, in a tsetse survey conducted in Tanzania, Lehane *et al.* (2000) failed to identify 13% of infections detected by microscopy using the available species-specific PCR. Similar studies have shown higher percentage of unidentified trypanosomes, 42% infected flies in Cameroon and Zimbabwe had unidentified trypanosomes (Morlais *et al.*, 1998b; Woolhouse *et al.*, 1996). The unidentified species may be pathogenic mammalian trypanosomes or more importantly, may be of zoonotic importance. These findings strengthened the need for a universal primer set for identification of species of both veterinary and zoonotic importance. The development of the ITS region as a universal target for the diagnosis of different trypanosome species occurring in either the vertebrate host or the invertebrate vector, aimed to decrease the number of species-specific PCR reactions required for diagnosis and allowed for the identification of new species infecting both the vector and vertebrate host.

The copy number of the rRNA locus varies from 100-200 copies per genome and each transcribed unit is composed of 18S, 5.8S and 28S rRNA genes as well as several ITS flanked by NTS. Eukaryotes have two ITS spacers, ITS1 which is located between the small subunit 18S gene and the large subunit 5.8S gene, and ITS2 which is located between the two large subunits 5.8S and 28S genes. The only species having an 18S rRNA G+C content of 55.4% (higher than other trypanosomes by 3%) is *T. vivax* (Stevens and Brisse, 2004).

Kin primers have been designed to react with kinetoplastid species, annealing in the conserved regions of the 18S and 5.8S rRNA to amplify the ITS1, the sequence of which is usually 300-800 bp in length (McLaughlin *et al.*, 1996). Desquesens *et al.* (2001) used Kin primers to evaluate their sensitivity in the detection of African livestock trypanosome DNA through a single PCR. The results showed the ability of the Kin primers to detect and discriminate all livestock trypanosome species through a single PCR reaction producing different amplified product for each species (Table 2.10). The sensitivity of kin primers to detect *T. congolense* Savannah was 85% of that of the species-specific PCR. In addition, the authors described that the sensitivity of Kin primers to amplify ITS1 region of *T. vivax* is low, attributing this to the fact that *T. vivax* target sequences have only 75 and 90% identity to the forward and reverse Kin primers, respectively. Moreover, with *T. vivax*, a non-specific product of 610 bp was observed and with the use of excess DNA, extra non-specific bands of more than 900 bp have been observed. By evaluating the detection of mixed DNA samples, all the PCR products expected were observed with mixing two to three of the DNA from *T. congolense* types together or with *T. vivax* or *Trypanozoon* DNA. It was interesting to notice that when mixing *T. brucei* s.l. and *T. vivax*, the *T. vivax* product sometimes became weak or indicating lower sensitivity for the detection of *T. vivax* DNA in the presence of *T. brucei* s.l. DNA. The authors recommended the requirement of improvement and investigation of new primers design in order to enhance the sensitivity of the test especially for detection of *T. vivax* DNA.

Table 2.10: Band sizes and Kin primers sequence (Desquesnes, 1997)

Trypanosome species		Obtained band size
T. brucei s.l.		540 bp
T. congolense Kilifi		680 bp
T. congolense Forest		780 bp
T. congolense Savannah		750 bp
T. vivax		305 bp
T. theileri		455 bp
T. simiae		435 bp
T. equiperdium		540 bp
T. evansi		540 bp
Primers	Kin1	5'-GCG TTC AAA GAT TGG GCA AT-3'
	Kin2	5'-CGC CCG AAA GTT CAC C-3'

Several attempts have been made to improve the design of primers amplifying the ITS1 locus for trypanosomes diagnosis using either semi-nested or nested PCR reactions. Geysen *et al*. (2003) stated that 18S ribosomal sub-unit is an ideal target sequence which is highly conserved, has sufficient polymorphism, and species specific sequences present in a multi-copy locus. The authors described a semi-nested PCR assay associated with restriction fragment length polymorphism assay (RFLP) using restriction enzymes to characterise all important bovine trypanosome species.

The use of PCR-RFLP was aimed to increase the sensitivity and specificity of the test and to distinguish different trypanosomes, because the amplified region encompasses such a polymorphic area in the 18S locus that specific restriction enzymes (*Msp1* and *Eco571*) can be found for each trypanosome species. The first amplification was done on the 18S gene using the forward primer 18ST nF2 and the reverse primer 18ST nR3. The second amplification was done using the forward primer 18ST nF2 and the reverse primer 18ST nR2 (Table 2.11). The primers were designed to amplify small sub-unit rRNA (ssu-rRNA) giving clear differences among the important trypanosome species infecting cattle.

Table 2.11: Band sizes and semi-nested PCR primers sequence (Geysen *et al.*, 2003)

Trypanosome species		**Obtained band size**
T. brucei s.l		660 bp
T. congolense Kilifi		711 bp
T. congolense Forest		674 bp
T. congolense Savannah		671 bp
T. vivax		616 bp
T. theileri		626 bp
T. simiae		629 bp
T. evansi		660 bp
Primers	18ST nF2	5'-CAA CGA TGA CAC CCA TGA ATT GGG GA-3'
	18ST nR3	5'-TGC GCG ACC AAT AAT TGC AAT AC-3'
	18ST nR2	5'-GTG TCT TGT TCT CAC TGA CAT TGT AGT G-3'

The primers showed a high target homology for all trypanosome sequences and increased the sensitivity 100-fold for *T. vivax* as compared to the test described by Desquesnes *et al*. (2001). The detection limit of the test on blood applied to filter papers in the case of single infections is one

trypanosome/40 µl blood. The authors documented that the inability to differentiate *T. brucei* s.l. from *T. evansi* (660 bp, each) is of less importance due to the geographical separation of their occurrence except in a few overlapping regions. Moreover, there was lack of differentiation between *T. simiae* and *T. theileri* using the two restriction enzymes. However, the authors reported that this had no repercussions for epidemiological surveys in cattle as *T. simiae* does usually not occur in cattle, whereas, this would be important in differentiating the trypanosomes infecting small ruminants and tsetse. This differentiation could be further investigated using *MboII* digestion of the same amplification product. The increases in cost and time, together with the low sensitivity, indicated the need of designing more specific primers targeting the ITS1 region with increased sensitivity (Geysen *et al.*, 2003). The same primers have been used by Delespaux *et al.* (2003) to evaluate their capacity in detecting mixed infections *in vitro* and *in vivo*. The results revealed that the restriction profiles of the different trypanosome species are easily recognised because the profile of each individual species is not overlapping with the profile of other species except for *T. congolense* and *T. theileri*. In this case, it is difficult to differentiate a single infection with *T. congolense* from a mixed infection of *T. congolense*/*T. theileri*.

Previously identified trypanosomes have been classified using primers designed to amplify the ssu rRNA region (Maslov *et al*., 1996). The primers were evaluated by Malele *et al*. (2003) who used the species-specific primers to confirm the identification of different trypanosomes in microscopically positive tsetse flies collected from Tanzania. The species-specific primers were able to identify 24% with trypanosome infection, whereas, a high proportion of the microscopically examined flies (about 75%) had unidentified trypanosome species. To investigate these unidentified samples, the authors used primers complementary to the conserved regions of trypanosomal ssu rRNA genes to amplify variable segments of the gene. Initial attempts using C and H primers resulted in recovery of ssu rRNA sequences with high similarity to Dipetran genes on database together with the desired trypanosomal genes. This was probably due to the close similarity of the primers to the tsetse ssu rRNA genes. Consequently, primer C was paired with primers (J and I) of low similarity to Dipetran ssu rRNA genes (Table 2.12).

Table 2.12: Primers sequence and PCR product length (Malele *et al.*, 2003; Maslov *et al.*, 1996)

Primer code	Primer sequence	Approximate length of PCR product (bp)		
		C-J	C-I	C-H
C	5'- CCG CGG TAA TTC CAG CTC C-3'	300	600	900
H	5'- CGT CAA TTT CTT TAA GTT TC-3'			
I	5'- GAC TAC AAT GGT CTC TAA TC-3'			
J	5'- CCA ACA AAA GCC GAA ACG GT-3'			

The amplified DNA fragments using the ssu rRNA primers were then cloned, sequenced and compared with ssu rRNA genes on database of known trypanosome species. The results revealed the identification of two new trypanosomes, one related to *T. vivax* and the other related to *T. godfreyi* (Malele *et al.*, 2003).

In Kenya, new ITS-based primers (ITS-CF an ITS-BR) have been evaluated for their use as universal set for trypanosomes diagnosis by amplifying ITS1 region (Njiru *et al*., 2005). Table 2.13 shows the sequence of ITS-CF and ITS-BR primers with the band size of the obtained products.

Table 2.13: Band sizes and ITS-CF, ITS-BR primers sequence (Njiru *et al.*, 2005)

Trypanosome species		Obtained band size
T. brucei s.l.		480 bp
T. congolense Kilifi		620 bp
T. congolense Forest		700 bp
T. congolense Savannah		700 bp
T. vivax		250 bp
T. simiae		400 bp
T. equoperdium		480 bp
T. evansi		480 bp
Primers	ITS-CF	5'-CCG GAA GTT CAC CGA TAT TG-3'
	ITC-BR	5'-TTG CTG CGT TCT TCA ACG AA-3'

The authors used serial dilutions of trypanosome DNA to test the sensitivity of the ITS-CF, ITS-BR and KIN primers together with the sensitivity of the species-specific primers, Table 2.14 summarises the results.

Table 2.14: Sensitivity of ITS based primers (CF and BR) and KIN primers compared with species-specific primers (Njiru *et al.*, 2005)

Trypanosome species	Sensitivity		
	Species specific primers	ITS (CF and BR)	KIN
T. evansi	0.1 pg*	10 pg	10 pg
T. brucei gambiesnse	0.1 pg	10 pg	10 pg
T. brucei rhodesiense	0.1 pg	10 pg	10 pg
T. congolense savannah	0.1 pg	10 pg	100 pg
T. congolense forest	0.1 pg	10 pg	100 pg
T. congolense kilifi	0.1 pg	10 pg	100 pg
T. godfreyi	10 pg	100 pg	1 ng
T. simiae	1 pg	100 pg	1 ng
T. vivax	1 pg	10 pg	1 ng

***(0.1 pg = one trypanosome, 1 pg = 10 trypanosomes, 10 pg = 100 trypanosomes, 100 pg = 1000 trypanosomes, 1 ng= 0.01 trypanosomes)**

Table 2.14 shows that the species-specific primers are more sensitive in trypanosomes detection than using rRNA target due to the higher copy number of the specific targets. Concerning the *T. congolense* subgroups, it is clear that ITS (CF and BR) primers are more sensitive compared with KIN primers in detecting the different *T. congolense* sub-species. In field application, 357 cattle samples collected from Kenya were examined using the different primer sets, for the samples positive using species-specific primers, ITS (CF and BR) were able to detect 84.9% compared with 67.4% using KIN primers. Although KIN primers were highly sensitive in detecting *T. vivax* (West African isolate) using the reference DNA, they were unable to detect any of the *T. vivax* species from field samples that were detected using species-specific primers and ITS (CF and BR).

From the above results, ITS (CF and BR) showed a higher diagnostic sensitivity than that previously described using KIN primers. The major difference between ITS (CF and BR) and KIN primers, is the ability of ITS (CF and BR) to detect *T. vivax* in field samples. This may be due to the 100% homology shown using ITS (CF and BR) with the available *T. vivax* sequence, while KIN primers showed only 75-90%. Despite the improved detection rates of *T. vivax* using ITS (CF and BR) primers, the authors identified only 77.4% of *T. vivax* from field samples in Kenya (East Africa). This fact may be related to low target DNA or the existence of *T. vivax* genetic variants (Masake *et al*., 1997; Masiga *et al*., 1992; Morlais *et al*., 2001).

The specificity of ITS (CF and BR) (no amplification with host and vector DNA) and capability of detecting all pathogenic trypanosomes in a single PCR indicates a great potential for this system as a universal test for pathogenic trypanosomes. However, the greatest challenge facing ITS-based tests is diagnostic sensitivity, because ITS1 has approximately 100-200 copies compared to 10,000 copies of species-specific tests targeting satellite DNA of *T. brucei* s.l. for instance (Desquesnes and Davila, 2002).

The development of a simple nested PCR method which detects the inter-specific length variation of the ITS regions of ribosomal genes producing a unique size of PCR product for each species has been reported by Cox *et al*. (2005). The authors compared the sensitivity of the nested ITS-PCR with the single PCR using KIN primers in detecting different trypanosomes using genomic DNA diluted with water and bovine blood. The sensitivity of KIN primers to detect trypanosomes using genomic DNA was found to be more than 70 trypanosomes/µl and 350-3000 trypanosomes/µl when whole trypanosomes were diluted with bovine blood. This indicates that blood has an inhibitory effect on PCR. However, the sensitivity of the nested ITS-PCR was a single trypanosome/µl using both the genomic DNA diluted in water and the whole trypanosome diluted in bovine blood.

The decision to use a nested PCR was based on increasing the sensitivity limit of the reaction with the increase in the target amount and potential dilution of the inhibitory factors (Cox, 2007). The nested ITS-PCR developed by Cox *et al*. (2005) has greatly simplified epidemiological studies involving cost reduction by a factor of four making the test a simple, cost effective, robust and reliable tool for investigating the complex epidemiology of African trypanosomiasis. Table 2.15 summarises the sequences of primers designed by Cox *et al*. (2005) and the amplified product size for each trypanosome.

Table 2.15: ITS1 primers sequence and band sizes (Cox *et al.*, 2005)

Trypanosome species			Obtained band size
T.congolense Forest			1513 bp
T.congolense Kilifi			1422 bp
T.congolense Savannah			1413 bp
T.congolense Tsavo			954 bp
T.brucei s.l.			1207-1224 bp
T.simiae			850 bp
T.vivax			611 bp
T.theileri			988 bp
Primers	Outer primers	ITS1	5'-GAT TAC GTC CCT GCC ATT TG-3'
		ITS2	5'-TTG TTC GCT ATC GGT CTT CC-3'
	Inner primers	ITS3	5'-GGA AGC AAA AGT CGT AAC AAG G-3'
		ITS4	5'-TGT TTT CTT TTC CTC CGC TG-3'

The aforementioned studies using generic primers to amplify the ITS1 region have concentrated on identifying only those trypanosomes causing the disease in cattle, whereas the range of trypanosomes infecting tsetse flies is far wider. Consequently, the study conducted by Adams *et al*. (2006) aimed to design generic primers in highly conserved areas to amplify the internal transcribed spacer (ITS) region of the rRNA repeat regions to enable species identification by inter-species size variation. The nested PCR developed is composed of two rounds of 35 cycles each using the outer primers in the first round and the inner primers in the second round. The produced band sizes varied from 150-750 bp according to the species (Table 2.16).

Table 2.16: ITS1 primers sequence and band sizes (Adams *et al.*, 2006)

Trypanosome species			Obtained band size
T. brucei s.l.			430 bp
T. congolense kilifi			560 bp
T. congolense Forest			640 bp
T. congolense Savannah			640 bp
T. vivax			180 bp
T. simiae Tsavo			380 bp
T. simiae			380 bp
T. grayi			380 bp
T. godfreyi			240 bp
Primers	Outer primers	TRYP1	5'-TGC AAT TAT TGG TCG CGC-3'
		TRYP2	5'-CTT TGC TGC GTT CTT-3'
	Inner primers	TRYP3	5'- AAG CCA AGT CAT CCA TCG-3'
		TRYP4	5'- TAG AGG AAG CAA AAG-3'

It is noticeable that some species have the same band size such as *T. congolense* Savannah/*T. congolense* Forest, *T. brucei* s.l/*T. evansi* and *T. simiae*/*T. grayi*. For tsetse examination, an additional species-specific PCR test is needed to distinguish these species. However, from my point of view, if this reaction is being used to screen samples collected from cattle, there is no need for further species-specific reaction to distinguish between the aforementioned species because it is easier to differentiate between these species depending on geographic distribution and the host range.

The sensitivity of this reaction is similar to that developed by Cox *et al*. (2005), both reported to detect 0.1 pg DNA (one trypanosome), but the sensitivity of the PCR test using the new generic primers (Adams *et al*., 2006) was reduced when dilution series with and without midgut tissues were compared. The authors collected wild tsetse samples from Tanzania in order to examine them using the new primers. The test was not able to identify a high proportion of samples (43.1%), they attributed this failure of identification to the presence of inhibitors in the midgut (proteases or haem-moieties) or that DNA storage method was not optimum. Moreover, the encountered failiure might be attributed to the unbounding of all trypanosome DNA effectively onto FTA®card matrix due to the presence of midgut material. The primers designed by Cox *et al*. (2005) were able to amplify the non pathogenic *T. theileri* which is transmitted mainly by Tabanids, while, the new primers designed by Adams *et al*. (2006) didn't amplify the ITS region for this trypanosome species because the authors were interested more with tsetse transmitted trypanosomes. The main drawbacks of using nested PCR are contamination issues related to the necessity of reagent and product handling before first round PCR, second round PCR and once again before gel electrophoresis (Boonma *et al.*, 2007).

As a further development in the diagnosis of trypanosomes using the length variation of multiple fragments within the 18S and 28S rRNA genes, Adams *et al*. (2008) have developed a new generic PCR method using fluorescent primers with the subsequent sizing of the products accurately and rapidly using an automated DNA sequencer. The authors have compared the use of the new fluorescent fragment length bar-coding (FFLB) (Hamilton *et al*., 2007) and the previously described method using ITS primers (Adams *et al*., 2006) in examining midgut samples collected from Tanzania. The comparison revealed that using ITS primers, 78% of the examined flies were identified to be infected with different trypanosome species, while, using FFLB, 97% were found to be infected. The authors documented that the FFLB system is accurate, quick and has an extremely high level of identification of infection. Moreover, FFLB revealed the existence of a putative new species of trypanosomes that is most related to *T. brucei* s.l. by phylogenetic analysis. However, the only obstacle of this new technique is the requirement of expensive equipment and trained technical staff for application.

In the current study, the different species-specific primers for the three major pathogenic trypanosmes *T. brucei* s.l., *T. congolense* Savannah and *T. vivax* are evaluated and compared to the use of ITS-PCR. The pan trypanosome ITS-PCR used in the current study is that developed by Cox *et al*. (2005).

3 Chapter three

Validation of different sample preparations for *Trypanosome* molecular diagnosis

3.1 Introduction

Polymerase chain reaction (PCR) has been used to amplify trypanosome DNA from genetic material isolated from both blood (Garcia *et al.*, 1995; Njiru *et al.*, 2004a) and buffy coat preparations (Mugittu *et al*., 2001). Rapid, efficient and reproducible procedures for isolating DNA before PCR amplification are essential for confirmation of infection (Alhassan *et al.*, 2007). However, the protocols to extract the DNA from samples are expensive, require specific instruments and kits and are time consuming. The transport of the collected fresh samples including blood, body fluids and tissues from animals and humans from the collection point to the laboratory may involve leakage risks because these samples are considered a potential biohazard to humans, animals or the environment. This is because they contain biological organisms such as viruses, bacteria, fungi and parasites and their toxins (WHO, 2005). Moreover, the transport of the samples may involve degradation of the genomic material due to temperature variation (Smith and Burgoyne, 2004).

Blood and buffy coat samples have been routinely collected on ordinary filter paper for analysis and detection of different blood pathogens such as *Theileria parva* (Geysen *et al.*, 1999), trypanosomes (Boid *et al.*, 1999; de Almeida *et al.*, 1998a; Delespaux *et al.*, 2003; Gonzales *et al.*, 2003; Goossens *et al.*, 2006; Katakura *et al.*, 1997; Mahama *et al.*, 2004), *Plasmodium* species (Wooden *et al.*, 1993) and human immunodeficiency virus (Panteleeff *et al.*, 1999.). Although collection of blood samples on filter paper for large scale sampling seems to be more convenient than other sampling procedures such as *in situ* DNA extraction (de Almeida *et al.*, 1998), these filter papers are not suitable for long term storage, as they do not protect the sample from spoiling and degradation (Smith and Burgoyne, 2004). For instance, in a study conducted by Katakura *et al*. (1997), blood samples from cattle collected on filter paper (stored at room temperature and at -20ºC for three months) were tested for the presence of trypanosome DNA by PCR. The authors observed 1000-fold decrease in the sensitivity of DNA detection from the filter paper stored at room temperature compared to those stored at -20ºC, confirming the inability of ordinary filter papers in protecting the genomic material for long periods when stored at room temperature.

Flinders Technology- Australia (FTA) technology has improved upon this paper based system. The FTA®card has been designed to fix and store nucleic acids directly from tissues, allowing the collection and archiving of nucleic acids (Belgrader *et al.*, 1995; Picard-Meyer *et al.*, 2007). The FTA matrix is impregnated with protein denaturants that cause lysis of cells and any organisms on contact. Moreover, these chemicals inhibit saprophytes during drying to ensure the safe handling of cards with out risk of biohazards (Belgrader *et al*., 1995; Rogers and Burgoyne, 1997). FTA technology also includes chelating agents and a free- radical trap designed to deal with atmospheric pollutants, thus protecting the entrapped nucleic acids for at least six years at room temperature (Rogers and Burgoyne, 1997). Although FTA®cards can protect DNA against acid gases and free radical generating pollutants in the atmosphere, it is reasonable to enhance the storage times of the DNA in

FTA®cards by inhibiting the atmospheric circulation by keeping them in specific envelopes with desiccants (Smith and Burgoyne, 2004). In conclusion, the advantages of using FTA®cards technology over other methods are due to the low sample volume (100 µl), safe handling, immobilization of the DNA, less storage space, ease of transportation and cheaper costs (Gutierrez-corchero *et al*., 2002).

FTA®cards have been used for blood storage (Devost and Choy, 2000; Gutierrez-corchero *et al.*, 2002), detecting bacterial pathogens (Lampel *et al*., 2000; Rogers and Burgoyne, 1997), detection of plant genes (Lin *et al*., 2000), detection of viral genomes (Ndunguru *et al*., 2005; Owor *et al*., 2007; Picard-Meyer *et al*., 2007) and in forensic human biology (Hsiao *et al*., 1999). The use of FTA®cards has been extended to include DNA detection from pathogenic protozoa and pathogenic organisms isolated from foods and clinical specimens using PCR (Orlandi and Lampel, 2000).

The feasibility of FTA®cards for the shipment, storage and detection of RNA rabies virus has recently been evaluated by Picard-Meyer *et al*. (2007). The authors concluded that the chemicals impregnated in the filter paper have made the samples no longer infectious; subsequently the samples do not induce any biohazards especially during shipment. Moreover, Owor *et al*. (2007) reported that the use of FTA®cards facilitates the large scale field sampling of viruses infecting plants, and yields DNA with sufficient stability, quantity and quality for further screening over time of storage at room temperature. The authors successfully extracted the viral DNA from FTA®cards stored at room temperature for nine months.

In trypanosome diagnosis, FTA®cards have been used for the storage of many materials including blood, buffy coat and extracted DNA for the detection of trypanosomes using PCR (Becker *et al*., 2004; Cox *et al*., 2005; Kaare *et al*., 2006; Picozzi *et al*., 2005; Picozzi *et al*., 2002). The use of FTA®cards for tsetse flies midgut storage has been documented by Adams *et al*. (2006, 2008), who noticed a lower trypanosome detection using PCR (56.9%) than was expected. They attributed this to different reasons including the sub-optimum binding of trypanosome DNA to the FTA®cards and the un-even binding of the trypanosome DNA throughout the card due to competition with midgut material. Also, the lower trypanosome detection was due to the high level of PCR inhibitors such as proteases and haem-moeties. The results have been improved reaching 78% after storage of the midgut samples in 100% ethanol and purification of DNA using ammonium acetate precipitation. Although FTA®cards are reliable medium for storage and transport of blood samples for PCR-based assays, long-term storage (>9 months) has been shown to prevent the complete removal of blood from discs by washing (Becker *et al*., 2004).

3.2 Objectives

The aim of this study is to validate different sample preparations in trypanosome diagnosis. This aim will be achieved by comparing different sample preparations collected in 2001-2002 by PCR in order to study the suitability of such preparations for the accurate diagnosis. The PCR screening of these

samples collected on FTA®cards will give an idea about the suitability of FTA®cards to sustain the integrity of DNA over long periods.

3.3 Study area

3.3.1 Overview

Uganda is a landlocked country in East Africa, bordered to the east by Kenya, to the north by Sudan, to the west by Republic of Congo and Tanzania to the south. The southern part of the country includes a substantial portion of Lake Victoria (Figure 3.1). The country is mostly plateau (900 meters altitude above the sea level and 800 kilometres inland from the Indian Ocean) and is suitable for both arable and livestock farming.

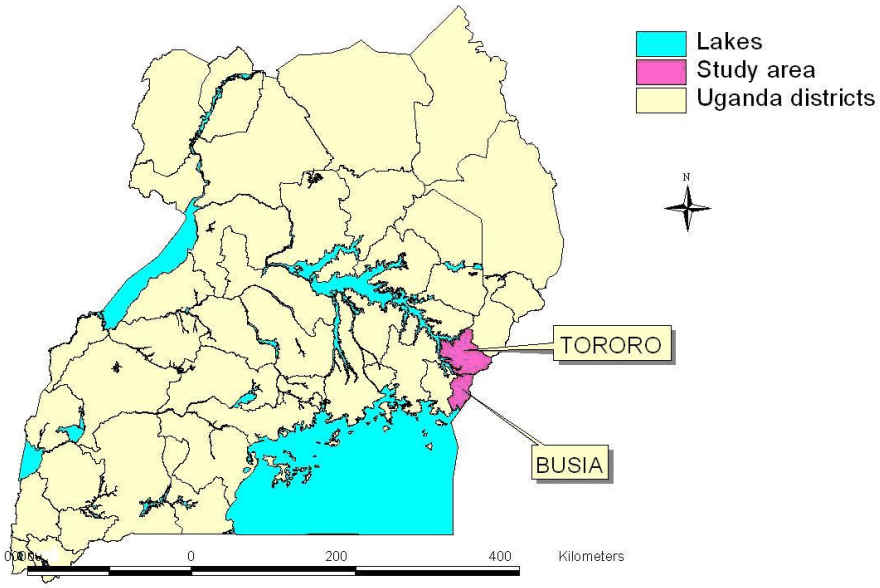


Figure 3.1: Uganda map with the studied two districts highlighted in purple

The two districts studied, Tororo and Busia, (highlighted in purple, Figure 3.1) are located in the East of Uganda. The main economic activity in both districts is agriculture. The predominant tsetse species in the study area is *Glossina fuscipes fuscipes*. *Glossina pallidipes* is present but to a lesser extent (Lancien *et al*., 1990).

3.3.2 Selected villages

3.3.2.1 Busia district villages

The selected villages include Buyimini village which lies to the east of the district. It is located a little further away from the nearest swamp compared to the other villages, with a surrounding forest cover. Kubo village lies in the mid-western part of the district, at the forest edge within the mixed farmland. It is also in close proximity to open Savannah grassland and a swamp. The third one is Nanjeho village, which lies to the southeast of the district; in close proximity to a swamp and a forest cover.

3.3.2.2 Tororo district villages

Three villages were selected from Tororo district; the first was Hitunga village lying to the north-west of the district at the edge of a swamp. It has open Savannah grassland with minimum forest cover. The second selected village was Magogi lying to the western part of the district in close proximity to a swamp with papyrus type of vegetation. It lies also in close proximity to woodland vegetation with trees of approximately four meters in height. The third village is Ojilai, which lies to the south-western part of the district in close proximity to a swamp with open savannah. Tropical high forests are also present to the north of the village.

3.3.3 Sample selection

The samples used in the current study were selected from samples collected within a longitudinal study carried out in Busia and Tororo districts – Uganda, in 2001 and 2002 by Kim Picozzi. For each animal, blood samples were collected for preparation of different materials including blood and buffy coat applied on FTA®cards and DNA extracted from whole blood and buffy coat preparations. Different sample preparations for 84 animals were selected in the current study to be analysed using PCR.

Selection of the samples in the current study was based on reported positive identification of infectious agent by microscopy, a reported low packed red cell volume (PCV), lower than 25, or a recorded poor condition score. The selection of these animals was also based on the availability of at least four sample preparations for each animal in order to make a comparison between these different preparations. The DNA samples were stored at -20ºC, while the FTA®card samples were stored at room temperature in envelopes with desiccant until the analysis was done in 2006 during the current research. Table 3.1, shows the number of cattle collected from each village. Figure 3.2, shows the types and numbers of different sample preparations analysed in the current study.

Table 3.1: The number of samples in each village

District	Village	Number of samples
Busia	Kubo	12
	Nanjeho	16
	Buyimini	4
Tororo	Hitunga	22
	Magogie	18
	Ojilai	12
Total		84

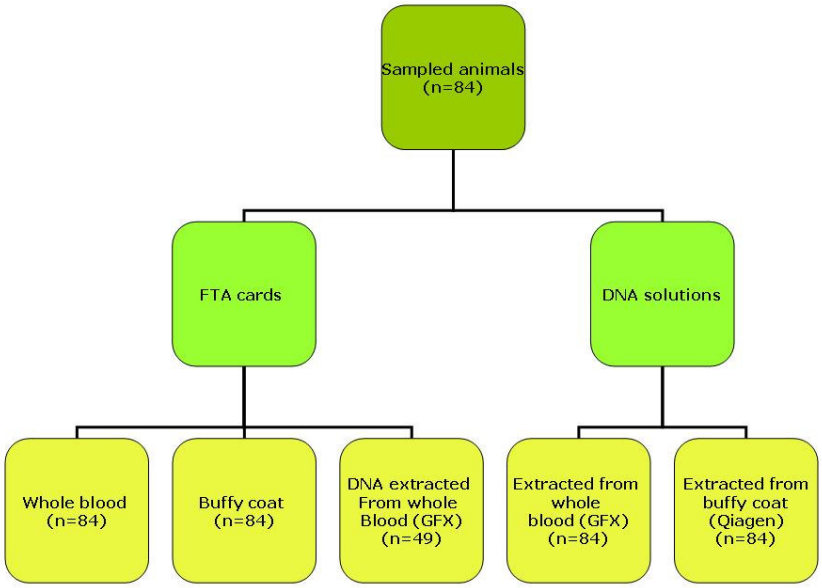


Figure 3.2: Different sample preparations collected from the selected animals (n: number of available material)

3.4 Material and methods

The samples used in the current study were collected and prepared by Kim Picozzi during a longitudinal study carried out in Busia and Tororo districts – Uganda, in 2001 and 2002.

3.4.1 Recording data

Data including the village name, age/sex/breed and body condition of the animal were recorded. Body condition scoring is a measure of the extent to which fat is stored or the muscle mass has declined. This can be measured by observing and examining the depth of the sub-lumbar fossa to assess the extent of emaciation (Nicholson and Butterworth, 1986). During sampling, Zebu and Ankole cattle were scored for the body condition as follows: Fat (F), Medium (M) or Lean (L).

3.4.2 Collection of heparinised whole blood

Blood was collected from the jugular vein into heparinised vacutainers for the preparation of buffy coat and for microscopic examination by thin and thick smears. Before the preparation of buffy coat samples, 100 µl of blood was spotted upon arrival to the laboratory on FTA®cards. Buffy coat samples were prepared by centrifugation of heparinised venous blood (4 ml) in a centrifuge at 3000 rpm. The buffy coat preparation was then collected from the interface between the packed red cells and the plasma and 100 µl was spotted on FTA®card for further analysis.

3.4.3 DNA preparation

3.4.3.1 Amershams GFX blood DNA purification kit

GFX purification uses a chaotropic agent (causes disruption of the molecular structure) to extract nucleated cells. The agent facilitates the binding of the genomic DNA to a glass fiber matrix contained in a MicroSpin column. Contaminants are removed with ethanolic washes and the purified DNA is eluted in a low ionic strength buffer.

Red Blood Cells (RBCs) lysis

To extract DNA from 300 µl whole blood, 900 µl RBC lysis solution (3x the sample volume) was added to 1.5 ml micro-centrifuge tube containing the blood sample. After inverting the tubes several times for thorough mixing, they were incubated at room temperature for five minutes. Centrifugation of the tubes at 12,000 rpm for 20 seconds was done to pellet the cells.

The supernatant was removed by aspiration without disturbing the white blood cell pellet. Some residual fluid remained on the sides of the tube to be used in re-suspending the cells before extraction.

Extraction

Vigorous vortexing of the cells in the residual supernatant (20-50 µl) was carried out to re-suspend them. Before the cells settled completely, 500 µl of extraction solution was added to the suspended cells. The mixture was then vigorously vortexed and incubated at room temperature for five minutes.

DNA binding

The entire extraction mixture was transferred to the GFX column and centrifuged at 8000 rpm for one minute. The flow-through was discarded by emptying the collection tube and then the GFX column was placed back inside the collection tube.

Washing

The DNA was extracted by adding 500 µl of extraction solution to the column. The mixture was centrifuged at 8000 rpm for one minute, and then the flow-through was discarded by emptying the collection tube. The contaminants were washed by adding 500 µl of wash solution and centrifugation of the mixture was carried out at 12,000 rpm for three minutes. The collection tube was discarded and the GFX column was transferred to a fresh 1.5 ml micro-centrifuge tube.

Elution

Pre-heated elution buffer (100 µl) was applied directly to the glass fiber matrix in the GFX column. The mixture was incubated at room temperature for one minute and then centrifuged at 8000 rpm for one minute to recover the purified DNA. The DNA yield using this method is 4.5-7.5 µg from 300 µl whole blood.

3.4.3.2 DNeasy®Blood and Tissue Extraction kit

For each sample, 200 µl buffy coat was collected from spinning down 1 ml whole blood. The DNA was extracted from this buffy coat amount by using DNeasy®Blood and Tissue Extraction kits (Qiagen).

Extraction

Proteinase K (20 µl) was pipetted into the bottom of a 1.5 ml micro-centrifuge tube containing 100 µl of buffy coat (each sample was divided into two and each half was processed separately). The volume was then adjusted to 200 µl with phosphate buffer solution (PBS). Extraction buffer (AL) was added in a volume of 200 µl, and then the tubes were thoroughly mixed by vortexing. Ethanol (200 µl) was added to the sample, the mixture was then vortexed thoroughly and pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifugation of the tubes was carried out at 8000 rpm for one minute, and then the flow-through and collection tubes were discarded.

Washing

The DNeasy Mini spin was placed in a new 2 ml collection tube. Contaminants were removed from the tubes by adding 500 µl washing buffer (AW1); the samples were then centrifuged at 8000 rpm for one minute. The same washing step was repeated but by using washing buffer (AW2) and centrifugation at 14,000 rpm for 3 minutes. The flow-through and collection tubes were discarded for the sample to be ready for the elution step.

Elution

The DNeasy Mini spin was placed in a clean 1.5 ml micro-centrifuge tube. DNA was eluted from the sample by pipetting 200 µl of the elution buffer (AE) directly on the DNeasy membrane. The tubes were incubated at room temperature for one minute, and then centrifuged at 8000 rpm for one minute. The final yield of DNA from 100 µl buffy coat sample is 3-12 µg.

3.4.4 FTA®cards

Preparation of the discs for the PCR reaction was done during the current study using the previously archived samples collected on FTA®cards during the longitudinal study in 2001-2002.

3.4.4.1 Preparation of discs from FTA®cards

For the three types of samples applied on FTA®cards, the same procedure for preparaing and washing the discs was applied. FTA®cards were placed on a cutting mat, using a puncher (Harris Micro Punch) a 0.2 mm disc was cut from the card. The number of discs to be examined were cut and placed together into a micro-centrifuge tube for washing. To avoid cross contamination between samples, an equivalent number of discs were taken from a blank filter paper after each sample.

3.4.4.2 Washing the discs

The discs were washed twice, 15 minutes each, in 200 µl (for each disc) of Whatman FTA purification reagent. This washing step removes materials such as haemoglobin which inhibits the PCR reaction, while the DNA is remained bound to the disc. The discs were then washed twice for 15 minutes in 200 µl (for each disc) 1 mM TE buffer (10mM Tris-HCL pH 8.0; 1mM EDTA pH 8.0) to remove the FTA purification reagent from the card. The discs were then transferred to PCR tubes and left to dry at room temperature for at least 90 minutes (Picozzi *et al.*, 2002). To detect any cross-contamination that may occur during sample washing, discs of a blank FTA®cards were washed with each batch of samples processed, and used as negative controls in the PCR.

3.4.5 Diagnosis by microscopy

Microscopic examination of heparinised blood samples for the presence of trypanosomes was done by Kim Picozzi using the haematocrit centrifugation technique (HCT) and buffy-coat phase contrast methods. The data of the microscopical examination was collected from the records of the longitudinal study conducted in 2001-2002.

3.4.5.1 Haematocrit centrifugation technique

Heparinised capillary tubes were filled with blood and one end of each tube was sealed with plasticine. The sealed capillary tubes were then centrifuged for four minutes at 12,000 rpm. After centrifugation, the capillary tubes were placed in a capillary tube holder along with a drop of immersion oil and examined under the microscope (Woo, 1970).

3.4.5.2 Buffy coat phase contrast method

Buffy coat samples were prepared by centrifugation of heparinised blood collected in capillary tubes. The capillary tube was cut with a diamond pointed pen 1 mm below the buffy-coat to include the uppermost layer of RBCs and 3 cm above to include the plasma. Using a micro-haematocrit capillary tube holder, the contents of the capillary tube were gently expressed on to a slide, mixed and covered with 22x22 mm cover slip. The slides were then examined by using phase contrast microscope at 250X magnification (Murray, 1977).

3.4.6 PCR

3.4.6.1 Primers and sizes of amplified products

The PCR reaction used in this chapter was Internal Transcribed Spacer PCR (ITS-PCR) to amplify ITS region of different trypanosome species. The primer sequences and the size of the amplified products related to each trypanosome species are summarised in Tables 3.2 and 3.3, respectively. The primers were re-suspended in water to a working concentration of 100 µM and stored frozen at -20ºC. The working stock was then diluted to a concentration of 10 µM before use and stored at 4ºC.

Table 3.2: Primer sequences for ITS-PCR reaction

Primer	Sequence		Reference
Outer primers	ITS1	5'-GAT TAC GTC CCT GCC ATT TG-3'	(Cox *et al*., 2005)
	ITS2	5'-TTG TTC GCT ATC GGT CTT CC-3'	
Inner primers	ITS3	5'-GGA AGC AAA AGT CGT AAC AAG G-3'	
	ITS4	5'-TGT TTT CTT TTC CTC CGC TG-3'	

Table 3.3: Amplified product sizes for each trypanosome species

Trypanosome species	Product size (bp)	Reference
T.congolense Forest	1513	(Cox *et al*., 2005)
T.congolense Kilifi	1422	
T.congolense Savannah	1413	
T.congolense Tsavo	954	
T.brucei s.l.	1207-1224	
T.simiae	850	
T.vivax	611	
T.theileri	988	

***ITS-PCR results with amplicon size more than 1400 bp were classified as *T. congolense* due to the difficulty in classifying the sizes of the three sub species**

3.4.6.2 The reaction conditions

The reaction volume of 25 µl contained the following components, Super Taq PCR buffer (HT Biotechnology Ltd, UK) (final concentrations of 10 mM Tri-HCL, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCL, 0.1% Triton X-100 and 0.01% (w/v) stabilizer), 0.2 µM of each outer primer ITS1 and ITS2, 1 mM total dNTP's (Bioline, London, UK) and 1.25 units of Biotaq (Bioline, London, UK). Trypanosome DNA was amplified from 1 µl of extracted DNA from whole blood and buffy-coat, while, one disc from samples applied on FTA®cards was used for the DNA amplification. One positive control (genomic DNA) and one negative control were run with each PCR.

The reaction conditions were as follows: 1 cycle of 95ºC for seven minutes followed by 35 cycles of 94ºC for one minute, 55ºC for one minute and 72ºC for two minutes. The reaction was carried out in a DNA Engine DYADTM Peltier Thermal Cycler.

For the ITS-PCR second round reaction, 1 µl of the PCR product from the first round reaction was placed in a fresh tube and 24 µl of the reaction mixture was added as detailed for the outer primers with the exception of the substitution of the outer primers with the inner primers ITS3 and ITS4. The reaction conditions were the same as in the first round.

3.4.7 Gel electrophoresis

3.4.7.1 Principle

The principle of gel electrophoresis is that at neutral pH, DNA has a negative charge, when it is loaded at the cathode, it moves toward the anode. The DNA fragments migrate through the gel with a shorter distance of migration of longer DNA fragments than that of short DNA fragments.

3.4.7.2 Method

Amplification products were resolved in 1% (w/v) agarose gels along with 100 bp molecular weight Superladder (ABgene, Epsom, Surrey, UK). The agarose gel was prepared in 1 x TBE (89 mM Tris-Borate, 2 mM EDTA, and pH 8.3) (Sigma-Aldrich, Poole, Dorset, UK) stained with 5 µM ethidium bromide which intercalates between DNA bases and fluoresces under Ultra Violet light. The gels were run in 1xTBE, 5 µM ethidium bromide for at least 45 minutes at 100 volts.

3.4.7.3 Visualisation

The amplified DNA bands were visualized under Ultra Violet transilluminator (Gel-Doc 2000, Bio-Rad). Exposure was optimised and pictures were stored with the aid of Bio-Rad Software (Quantity One, Bio-Rad).

When the amplification reaction gave a signal of the expected size (Table 3.3) according to the set of primers used without any signal in the negative control, infection was considered to be confirmed. A positive control was used to be sure that the reaction mixture was working. Figure 3.3 shows gel picture of positive ITS-PCR results.

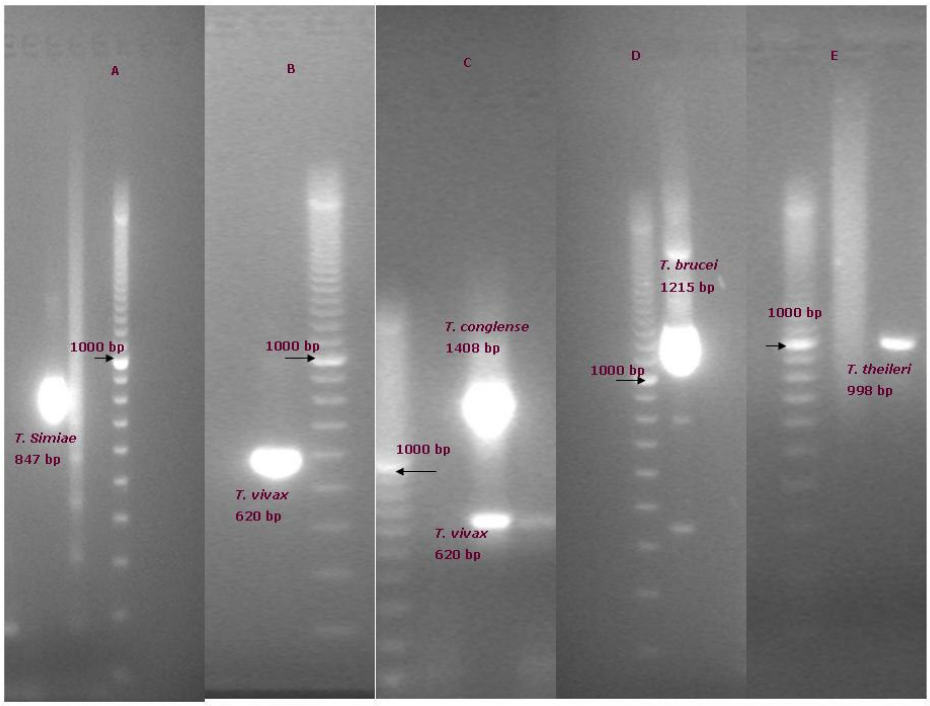


Figure 3.3: Example of ITS-PCR results in 1% agarose (A: *T. simiae*, B: *T. vivax*, C: *T. congolense/T. vivax* mixed infection, D: *T. brucei* s.l., E: *T. theileri* (100 bp ladder)

3.4.8 Statistical analysis

The difference in trypanosome detection between the different sample preparations used was analysed using Chi-squared test (degree of freedom were noted as a subscript to the χ^2 –statistic) that was computed using Minitab version 15 (Minitab, Inc.). Differences were considered to be significant at $p<0.05$. For the tested values containing expected frequencies of less than five, Fisher's exact test was used instead of Chi-squared test.

The sensitivity of a test is defined as the proportion of true positives detected by the test; representing the probability of the test to produce true positive result when compared with the gold standard (reference test). On the other hand, specificity of a test is the proportion of true negatives that are detected by the test; representing the probability of the test to produce true negative result with the gold standard.

The positive predictive value of a test is the probability that the positive result is a true positive. While, the negative predictive value of the test is the probability that the negative result is a true negative. Unlike sensitivity and specificity, the predictive value of a test varies depending upon the prevalence of the condition being tested in a specific population.

Table 3.4 shows the outcomes of a test in relation to the gold standard and the equations to calculate the sensitivity, specificity, positive predictive value and negative predictive value (Thrusfield, 1986).

Table 3.4: Outcomes of a diagnostic test and equations (Thrusfield, 1986)

		Gold standard		
Test		Positive	Negative	Total
	Positive	TP	FP	TP+FP
	Negative	FN	TN	FN+TN
	Total	TP+FN	FP+TN	TP+FP+FN+TN
Equations				
	Sensitivity= TP/(TP+FN)			
	Specificity= TN/(FP+TN)			
	Positive predictive value (PPV)= TP/(TP+FP)			
	Negative predictive value (NPV)= TN/(TN+FN)			

TP: True positive, FP: False positive, TN: True negative, FN: False negative

The Kappa value was used to determine the level of agreement between the diagnostic test and the gold standard. The Kappa value lies between 0 and 1, where 0.8-1 means a very good agreement between tests and 0 means that the association is no better than expected from chance alone. Kappa value >0-0.2 indicates poor agreement, >0.2-0.4 indicates fair agreement, >0.4-0.6 means moderate agreement and >0.6-0.8 means good agreement. A negative Kappa value indicates that the two tests agree less than would be expected by chance (Altman, 1991; Avila *et al.*, 1991). Kappa value is calculated by the formula: (A-E)/ (N-E) where A is the sum of agreements defined as the sum of positives and negatives common to both techniques. E is the sum of expected occurrences defined as the sum of all positives divided by N plus the sum of all negatives divided by N. While, N is the

number of animals examined by both methods. The agreement of test results was based on the analysis of the Kappa statistics using the computer programme Win Episcope 2.0.

Kappa value= (A-E)/ (N-E)

A=∑all positives and negatives common to both techniques

E= ((∑positives)/N+ (∑negatives)/N)

N= the total number of animals examined by both techniques.

3.5 Results

In 2001/2002; a longitudinal study was carried out to observe the impact of block treatment of cattle with drugs against trypanosomiasis and East Coast Fever (ECF). At the last collection point, both DNA and blood samples were collected. This chapter focuses on 84 animals selected on the basis of reported positive identification of infectious agent by microscopy, a reported low PCV (lower than 25) or a recorded poor condition score. These animals were also selected on the basis of the existence of at least four analytical samples namely whole blood on FTA®cards and DNA extracted directly from whole blood, and buffy coat on FTA®cards as well as DNA extracted from buffy coat. For 49 animals, DNA was also stored on FTA®cards. This information is summarised in Figure 3.2.

3.5.1 Data collected from microscopical examination of the selected animals

The microscopy results obtained from the records of the longitudinal study indicated a total of 15.5% animals were infected with different trypanosome species in single and mixed infection. Table 3.5 summarises the proportion of different trypanosome species identified by microscopy in the examined animals.

Table 3.5: Proportion of trypanosome species detected by microscopy [% (fraction; 95% CI)]

Number of animals examined	Parasitic events*			Proportion of infected animals
	T. brucei s.l.	*T. congolense*	*T. vivax*	
84	2.4% (2/84; 0.3-8.3)	1.2% (1/84 ;0.03-6.5)	13.1% (11/84 ;5.9-20.8)	15.5% (13/84; 8.5-25)

***Includes parasite species existing in single and mixed infections**

The results in Table 3.5 show the proportion of parasitic events identified by microscopy; in the case of mixed infection with more than one species, the parasites in the combination were categorised according to the species. For instance, an animal infected with *T. brucei* s.l./*T. vivax* mixed infection, *T. brucei* s.l. was included in the column of *T. brucei* s.l. single parasitic event and *T. vivax* was

included in the column of *T. vivax* single parasitic event. This explains why the overall incidence of the parasitic events exceeds the overall infected animals.

The results show that the proportion of *T. brucei* s.l., *T. congolense* and *T. vivax* diagnosed by microscopy, was 2.4%, 1.2% and 13.1%, respectively. The identification of *T. vivax* by microscopy was significantly higher than *T. brucei* s.l. (χ_1^2=6.8, p=0.009) and *T. congolense* (χ_1^2=8.7, p=0.003).

3.5.2 PCR results from examining the selected animals

The PCR reaction used in this study was ITS-PCR which targets internal transcribed spacer in the trypanosome genome. The reaction was used to amplify trypanosome genomic material from different sample preparations collected from 84 selected animals (Figure 3.2). The preparations included examining five discs from each material applied on FTA®cards; each disc was examined by a separate ITS-PCR. Moreover, 1 µl from DNA extracted from both whole blood and buffy coat was also examined.

For the PCR results, the true infection status of the 84 animals, was defined as positive if at least one of the different examined materials was positive by PCR (Picozzi *et al*., 2002). ITS-PCR identified 72.6% of the sampled animals to be infected with different trypanosome species including single and mixed infections with pathogenic (*T. brucei* s.l., *T. congolense* and *T. vivax*) and non-pathogenic species (*T. theileri*). Table 3.6 shows the proportion of parasitic events diagnosed by ITS-PCR of different sample preparations from the selected animals.

Table 3.6: Proportion of trypanosome species detected by ITS-PCR [% (fraction; 95% CI)]

Number of animals examined	Parasitic events*				Proportion of animals infected with any trypanosomes	Proportion of animals infected with pathogenic trypanosomes
	T. brucei s.l.	*T. congolense*	*T. vivax*	*T. theileri*	72.6% (61/84; 61.8-81.8))	27.4% (23/84; 18.2-38.2)
84	13.1% (11/84; 6.7-22.2)	7.1% (6/84 ; 2.7-14.9)	8.3% (7/84 ; 3.4-16.4)	53.6% (45/84 ;42.4-64.5)		

***Includes parasite species existing in single and mixed infections**

The results in Table 3.6 show the proportion of parasitic events diagnosed by ITS-PCR. The proportion of *T. brucei* s.l., *T. congolense*, *T. vivax* and *T. theileri* diagnosed by PCR was 13.1%, 7.1%, 8.3% and 53.6%, respectively. The prevalence of *T. theileri* species identified by ITS-PCR was significantly higher than the other pathogenic species (χ_1^2=10.8, p=0.001).

Excluding the non-pathogenic species *T. theileri* from the overall prevalence of the parasitic events would help to precisely compare the level of PCR sensitivity with microscopy. This exclusion was

based on the inability of microscopy to identify this species due to characteristic low parasitemia level.

3.5.3 Evaluation of PCR versus microscopy in trypanosome diagnosis

Table 3.7 shows the results of microscopy compared to ITS-PCR in identifying animals infected with trypanosomes.

Table 3.7: ITS-PCR versus microscopy in diagnosing animals infected with trypanosomes

Microscopy		ITS-PCR					
		All trypanosome species			Pathogenic trypanosomes		
		Positive	Negative	Total	Positive	Negative	Total
	Positive	12	1	13	6	7	13
	Negative	49	22	71	17	54	71
	Total	61	23	84	23	61	84

The results in Table 3.7 show that comparing PCR and microscopy in detecting any trypanosome species (pathogenic and non-pathogenic) 12 animals were found to be positive for trypanosomes by both microscopy and PCR, 49 were positive by PCR but negative by microscopy. One animal was identified by microscopy to have infection but missed by PCR, while 22 animals were found negative by both methods.

The detection of trypanosomes in the examined animals by either microscopy or ITS-PCR was compared to a gold standard (defined as a positive PCR and/or microscopy result in each examined animal) and tested for the agreement using Kappa test. The sensitivity of microscopy in detecting any trypanosomes compared to the gold standard was found to be 21% (95% CI: 10.8-31.1), while the sensitivity of ITS-PCR compared to the gold standard was found to be 98.4% (95% CI: 95.3-100). The diagnostic predictive value showed that the probability of microscopy detecting animals negative for trypanosomes was 31% (95% CI: 20.2-41.7). The Kappa value was determined to be 0.1 indicating poor agreement between the gold standard and microscopy in identifiying any trypanosome infections. The negative diagnostic predictive value of ITS-PCR was calculated to be 95.7% (95% CI: 87.3-100) compared to the gold standard with a Kappa value of 0.9 indicating very good agreement. The results show also that PCR proving to be significantly 4.7 times more sensitive than microscopy in identifying any trypanosomes (χ_1^2=72.1, p<0.001).

Looking at the overall prevalence of pathogenic species results after excluding the non-pathogenic *T. theileri*, the sensitivity of microscopy in identifying pathogenic species was 43.3% (95% CI: 25.6-61.1) compared to the gold standard. The negative predictive value of microscopy for diagnosing animals infected with pathogenic trypanosomes was 76.1% (95% CI: 66.1-86). ITS-PCR was shown to be 1.7 times more sensitive than microscopy in diagnosing pathogenic species with 76.7% (95% CI:

61.5-91.8) sensitivity compared to the gold standard. However, this was not statistically significant (χ_1^2=3.5, p=0.06). The negative diagnostic predictive value of ITS-PCR was calculated to be 88.5% (95% CI: 80.5-96.5); ITS-PCR did not detect seven infected animals diagnosed positive by microscopy. The Kappa value for the agreement between PCR and the gold standard in detecting pathogenic trypanosomes was 0.8 (very good agreement) while 0.5 for microscopy (moderate agreement).

For more specific evaluation of ITS-PCR and microscopy sensitivity in the diagnosis of trypanosomes, the data were further broken down to compare the two protocols in diagnosing each species. Table 3.8 shows the results comparing ITS-PCR and microscopy in diagnosing *T. brucei* s.l., *T. congolense* and *T. vivax*.

Table 3.8: ITS-PCR versus microscopy in diagnosing animals infected with pathogenic trypanosomes

Microscopy		**ITS-PCR**								
		T. brucei s.l.			*T. congolense*			*T. vivax*		
		Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
	Positive	0	2	2	1	0	1	4	7	11
	Negative	11	71	82	6	77	83	3	70	73
	Total	11	73	84	7	77	84	7	77	84

The results in Table 3.8 show that there was less agreement than would be expected by chance between ITS-PCR and microscopy in detecting *T. brucei* s.l. species (Kappa value=-0.04). Moreover, poor agreement was observed between the two tests in diagnosing *T. congolense* and *T. vivax*, Kappa value was 0.2 and 0.4, respectively. Sensitivity of ITS-PCR in amplifying *T. congolense* genomic material was seven times higher than the detection by microscopy (p=0.06) and this was statistically insignificant. While, the sensitivity of microscopy in detecting *T. vivax* was 1.7 times higher than the identification of the species using ITS-PCR but this was statistically insignificant (χ_1^2=0.99, p=0.3).

3.5.4 Suitability of various sample preparations for the accurate diagnosis of trypanosome infected animals

3.5.4.1 Diagnosis of any trypanosome species

A total of 84 animals were examined for the presence of trypanosome DNA in different sample preparations using ITS-PCR. The sample preparations included whole blood, buffy coat and DNA applied on FTA®cards, also, extracted DNA from blood and buffy coat in homogenous solution (Figure 3.2).

Using a gold standard (defined as positive PCR result from amplifying trypanosome DNA from FTA®cards and/or positive PCR result from amplifying trypanosome DNA in homogenous solutions),

72.6% (61/84; 95% CI: 61.8-81.8) animals were diagnosed to be infected with any trypanosome species. Compared to the gold standard, the sensitivities and negative predictive values of the examined preparations were determined as shown in Table 3.9.

3.9: Sensitivity and NPV of the examined preparations compared to the gold standard for identifying any trypanosome species (n=84)

Material	True positive	False positive	True negative	False negative	% NPV (95%CI)	% Sensitivity (95%CI)
Whole blood onFTA®cards	3	0	23	58	28.4 (18.6-38.2)	4.9 (0-10.3)
Buffy coat on FTA®cards	3	0	23	58	28.4 (18.6-38.2)	4.9 (0-10.3)
DNA on FTA®cards*	4	0	17	28	37.8 (23.6-51.9)	12.5 (1.1-24)
DNA extract (GFX)	53	0	23	8	74.2 (58.8-89.6)	86.9 (78.4-95.4)
DNA extract (Qiagen)	37	0	23	24	48.9 (34.6-63.2)	60.7 (48.4-72.9)

***DNA samples applied on FTA®cards were only 49 samples**

Table 3.9 shows the sensitivities and negative predictive values of different sample preparations in diagnosing any trypanosome using ITS-PCR. The results show that the sensitivity of trypanosome genomic material amplification from DNA homogenous solution extracted from whole blood (GFX) was 17.7 times higher than the amplification of the same whole blood extracted DNA from FTA®card matrix, which was statistically significant (χ_1^2=35.9, p<0.001). Also, the amplification sensitivity of genomic materials in DNA solution extracted from buffy coat preparation (Q) was 12.4 times higher than amplification from buffy coat on FTA®cards which was significant (χ_1^2=35.7, p<0.001). Moreover, the sensitivity of detecting trypanosomes from DNA solutions extracted from whole blood (GFX) was 1.4 times higher than amplifying the DNA from buffy coat extract (Q), which was statistically significant (χ_1^2=6.1, p=0.01). The sensitivity of amplifying any trypanosome genomic material from DNA extract applied on FTA®cards was 2.6 times higher than from whole blood and buffy coat applied on FTA®cards, this was not statistically significant (p=0.4).

The results in Table 3.10 summarise the Kappa statistic that gives a measurement of the degree of agreement between the studied materials compared to the gold standard for the amplification of any trypanosome DNA using ITS-PCR.

Table 3.10: Kappa value for testing the agreement between detecting trypanosome DNA in different sample preparations

Material	Kappa value	Interpretation
Whole blood on FTA®cards	0.03	Poor agreement
Buffy coat on FTA®cards	0.03	Poor agreement
DNA on FTA®cards	0.1	Poor agreement
DNA extract (GFX)	0.8	Very good agreement
DNA extract (Qiagen)	0.5	Moderate agreement

The results in Table 3.10 show that there was a very good agreement between detecting trypanosome genomic material in DNA extract from whole blood compared to the gold standard. Poor agreement was detected when the same extract was applied on FTA®cards compared to the gold standard. A moderate agreement in amplifying trypanosome ITS target was also detected between DNA extract from buffy coat and gold standard. However, poor agreement was observed in detecting any trypanosome DNA from whole blood and buffy coat applied to FTA®cards compared to the gold standard.

3.5.4.2 Diagnosis of pathogenic trypanosome species

Excluding the non-pathogenic trypanosome species *T. theileri* would give more precise evaluation of the suitability of different material preparations for the diagnosis of the three important pathogenic African trypanosomes. This approach omitted 38 animals previously identified as infected with *T. theileri*. In this case, the gold standard (defined as positive PCR result from amplifying pathogenic trypanosome DNA from FTA®cards and/or positive PCR result from amplifying pathogenic trypanosome DNA in homogenous solutions), detected 27.4% (23/84; 95% CI: 18.2-38.2) animals infected with pathogenic trypanosomes. Compared to the gold standard, the sensitivities and negative predictive values of the examined preparations were determined as shown in Table 3.11.

Table 3.11: Sensitivity and NPV of the examined preparations compared to the gold standard for identifying pathogenic species (n=84)

Material	True positive	False positive	True negative	False negative	% NPV (95%CI)	% Sensitivity (95%CI)
Whole blood onFTA®cards*	0	0	61	23	NA	NA
Buffy coat on FTA®cards	1	0	61	22	73.5% (64-83)	4.3% (0-12.7)
DNA on FTA®cards**	4	0	27	18	60% (45.7-74.3)	18.2% (2.1-34.3)
DNA extract (GFX)	18	0	61	5	92.4% (86.1-98.8)	78.3% (61.4-95.1)
DNA extract (Qiagen)	11	0	61	12	83.6% (75.1-92.1)	47.8% (27.4-68.2)

***Sensitivity was not calculated due to the inability of the PCR to amplifiy any DNA from this material ** DNA samples applied on FTA®cards were only 49 samples**

Table 3.11 shows the sensitivities and negative predictive values of different sample preparations in diagnosing pathogenic trypanosomes using ITS-PCR. The results show that the sensitivity of amplifying pathogenic trypanosome genomic material from whole blood DNA extract (GFX) was 78.3% compared to the gold standard, however, the same DNA extract applied on FTA®cards failed to be amplified using ITS-PCR.The sensitivity of amplifying pathogenic trypanosomes from whole blood extract (GFX) was 1.6 times higher than amplification from buffy coat extract (Q), which was statistically insignificant (χ_1^2=1.5, p=0.2). Buffy coat DNA extract (Q) was 11 times more sensitive in detecting pathogenic trypanosomes than from buffy coat applied on FTA®cards which was statistically significant (χ_1^2=7.3, p=0.01). Comparing the materials applied on FTA®cards in amplifying pathogenic trypanosome DNA, whole blood extract (GFX) on FTA®cards was 4.2 times higher than from buffy coat applied on FTA®cards, which was statistically insignificant (p=0.06). However, screening five discs from whole blood on FTA®cards failed to amplify any of the pathogenic trypanosome DNA.

The results in Table 3.12 summarise the Kappa statistic that gives a measurement of the degree of agreement between each of the studied materials and the gold standard for the amplification of pathogenic trypanosome DNA.

Table 3.12: Kappa value for testing the agreement

Material	Kappa value	Interpretation
Whole blood on FTA®cards	NA	NA
Buffy coat on FTA®cards	0.1	Poor agreement
DNA on FTA®cards	0.2	Poor agreement
DNA extract (GFX)	0.8	Very good agreement
DNA extract (Qiagen)	0.6	Very good agreement

The results in Table 3.12 show that there was a very good agreement between detecting trypanosome genomic material in DNA extract from whole blood compared to the gold standard. Poor agreement was detected when the same extract was applied on FTA®cards; compared to the gold standard. A moderate agreement in amplifying trypanosome ITS target was also detected between DNA extract from buffy coat and gold standard. Moreover, poor agreement was noticed in detecting the pathogenic trypanosome DNA from buffy coat samples applied on FTA®cards compared to the gold standard.

3.5.5 Proportion and species of trypanosomes detected by ITS-PCR in the examined materials

The proportion and species of trypanosomes identified using PCR amplification of DNA from different materials are summarised in Table 3.

Table 3.13: Proportion and species of trypanosomes detected in different examined materials

Examined Materials	Parasitic events*				Mixed infection including *T. theileri*	Mixed infection excluding *T. theileri*
	T. brucei s.l.	*T. congolense*	*T. vivax*	*T. theileri*		
Blood on FTA®cards	0 (0/84; 0-3.5)	0 (0/84; 0-3.5)	0 (0/84; 0-3.5)	3.6% (3/84; 0.7-10.1)	0 (0/84; 0-3.5)	0 (0/84; 0-3.5)
Buffy coat on FTA®cards	1.2% (1/84; 0.03-6.5)	0 (0/84; 0-3.5)	0 (0/84; 0-3.5)	2.4% (2/84; 0.3-8.3)	0 (0/84; 0-3.5)	0 (0/84; 0-3.5)
DNA on FTA®cards	0 (0/49; 0-5.9)	0 (0/49; 0-5.9)	8.2% (4/49; 2.3-19.6)	0 (0/49; 0-5.9)	0 (0/49; 0-5.9)	0 (0/49; 0-5.9)
DNA extract (GFX)	9.5% (8/84; 4.2-17.9)	7.1% (6/84; 2.7-14.9)	6% (5/84; 2-13.3)	45.2% (38/84; 34.3-56.5)	4.8% (4/84; 1.3-11.7)	1.2% (1/84; 0.03-6.5)
DNA extract (Qiagen)	7.1% (6/84; 2.7-14.9)	3.6% (3/84; 0.7-10.1)	2.4% (2/84; 0.3-8.3)	34.5% (29/84; 24.5-45.7)	3.6% (3/84; 0.7-10.1)	0 (0/84; 0-3.5)
Gold standard**	13.1% (11/84; 6.7-22.2)	7.1% (6/84; 2.7-14.9)	8.3% (7/84; 3.4-16.4)	53.6% (45/84; 42.4-64.5)	9.5% (8/84; 4.2-17.9)	1.2% (1/84; 0.03-6.5)

***Includes parasite species existing in single and mixed infections **Gold standard is defined as animals diagnosed positive using ITS-PCR on at least one of the sample preparations used**

The results in Table 3.13 show that *T. congolense* genomic material was not amplified from materials applied on FTA®cards. However, this species was diagnosed only in the DNA extract preparations. *T. brucei* s.l. was significantly detected in DNA extract solutions rather than in FTA®card preparations (p=0.03). *T. vivax* was only detected in FTA®card containing DNA extract and this was insignificantly different from diagnosing this species in the same extract when tested using only 1 µl for PCR.

Mixed infections were only detected in the DNA extract. A total of eight animals were found to harbour a mixed infection, with *T. theileri* included in seven of them. Screening of five discs from whole blood and buffy coat applied on FTA®cards failed to detect any type of mixed infection. Therefore, 30 discs from material applied on FTA®cards of animals infected with mixed infection

were screened further. There was not enough material to screen 30 discs more from DNA extract applied on FTA®cards. Table 3.14 shows the results from screening 30 discs contained whole blood and buffy coat compared to PCR results using only 1 µl DNA extract from whole blood and buffy coat from animals with mixed infections.

Table 3.14: Further screening of animals with mixed infection

Sample number	1 µl DNA extract from whole blood (GFX)	30 discs from whole Blood on FTA card (positive discs)	1 µl DNA extract from buffy coat (Q)	30 discs from buffy coat on FTA card (positive discs)
1	*T. brucei* s.l./ *T. theileri*	0	*T. brucei* s.l./ *T. theileri*	0
2	*T. theileri/ T. vivax*	0	0	(2/30) *T. theileri*
3	*T. theileri/ T. vivax*	0	*T. theileri*	0
4	*T. vivax*	(7/30) *T. theileri*	*T. theileri/ T. vivax*	(8/30) *T. theileri*
5	*T. brucei* s.l.	0	*T. brucei* s.l./ *T. theileri*	0
6	*T. brucei* s.l./ *T.congolense*	0	*T. brucei* s.l	0
7	*T. brucei* s.l./ *T. theileri*	0	*T. brucei* s.l./ *T. theileri*	0
8	*T. theileri*	0	*T. brucei* s.l	0

The results in Table 3.14 show that mixed infections with different trypanosome species were only diagnosed in extracted DNA solutions either from whole blood or buffy coat. In spite of examining 30 punches from both whole blood and buffy coat applied on FTA®cards, mixed infection could not be detected.

3.6 Discussion

3.6.1 Evaluation of PCR and microscopy in trypanosomes diagnosis

Diagnosis of trypanosomiasis using molecular methods has been shown to be highly sensitive and more specific than the more traditional microscopic techniques (Desquesnes, 1997; Masake *et al*., 1997; Masake *et al*., 2002). Despite this fact, the application of the molecular techniques in the disease control and research programmes in the field has not been widely used (Clausen *et al.*, 1998; Masake *et al.*, 1997; Mugittu *et al.*, 2001; Solano *et al.*, 1999). This is due to the costs, the need of specialised equipment and the high time costs involved in performing molecular techniques.

In the present study, a total of 84 cattle were examined for any trypanosome species by microscopy and PCR amplification on different sample preparations from each animal. The results showed that microscopy was able to detect trypanosome infections in 13 (15.5%) animals while PCR detected 61 (72.6%). PCR was 4.7 times more sensitive compared to microscopy when compared to one another, the agreement between these two diagnostic approaches was poor (Kappa value=0.1).

3.6.1.1 *T. theileri* species

T. theileri is a cosmopolitan parasite of cattle which has been reported from cattle in every country they inhabit (Farrar and Klei, 1990; Herbert, 1964; Matthews *et al*., 1979; Rodrigues *et al*., 2003; Schlafer, 1979; Verloo *et al*., 2000). This parasite is the species of the subgenus Megatrypanum that is produced in the hindgut of the vector then transmitted to the host through faecal contamination (Hoare, 1972; Levine *et al*., 1980). The most important vectors for transmitting this parasite are Tabanids (Dirie *et al*., 1990; Marquardt *et al*., 2000). In a study conducted by Bose and Heister (1993), 39% of the collected Tabanid flies were found to be carrying *T. theileri*. Moreover, ticks were found to have a high vectorial capacity for *T. theileri* suggesting the ability of ticks to act as a vector for the parasite (Latif *et al*., 2004). Although considered non pathogenic, other cases of clinical illness suggested that *T. theileri* may be considered potentially pathogenic, showing a correlation of this species with lymphocytosis and suggesting an activation of its virulence in the presence of some intercurrent infections, stress, gestation and poor nutritional status (Doherty *et al.*, 1993; Hussain *et al.*, 1985; Rodrigues *et al.*, 2003; Seifi, 1995).

Although the large size of the parasite aids in distinguishing from other trypanosome species present in the same microscopic field, it was not detected by microscopy in this study. This was attributed to the low parasitaemia caused by *T. theileri* (Dirie *et al*., 1990) making the detection of the parasite by microscopy difficult due to the lower number of parasites than the microscopy threshold. Similar results were obtained by Goossens *et al*. (2006) who diagnosed 42.4% of the examined cattle to be infected with *T. theileri* species using PCR, while using microscopy, 3.1% of the examined cattle were positive for *T. theileri*. The high prevalence of *T. thieleri* in the studied area indicated the presence of other blood sucking arthropods, mainly Tabanids (Dirie *et al*., 1990; Marquardt *et al*., 2000; Rodrigues *et al*., 2003), that may act also as a vector for other trypanosomes such as *T. vivax* by mechanical transmission (Desquesnes and Dia, 2003; Desquesnes and Dia, 2004). Similar findings from Tororo district were reported by Cox (2007) who analysed 35 blood samples applied on FTA®cards for the prevalence of different trypanosome species using ITS-PCR. The results revealed that *T. theileri* was the predominant species (25.7%) and it was involved in all mixed infections reported.

3.6.1.2 Overall pathogenic trypanosomes

Analysing the same data after excluding the non-pathogenic *T. theileri* indicated that ITS-PCR was 1.8 times more sensitive than microscopy in diagnosing pathogenic trypanosomes.

Microscopy missed 17 cases found to be positive for pathogenic trypanosomes by PCR amplification (Table 3.7) indicating that the use of microscopy in diagnosing animal trypanosomiasis does not give the actual disease situation, leading to under estimation of the herd prevalence. These findings are in agreement with those reported by Clausen *et al*. (1998) who evaluated the sensitivity and specificity of

PCR assay compared with microscopy in detecting trypanosomes in infected cattle, Uganda. The results revealed that the detection rate of PCR (34.8%) was three times higher as compared to microscopy. In another study conducted in Tororo and Busia districts where the current study was also done, McOdimba (2006) reported that PCR was 6.4 times more sensitive than microscopy in identifying different pathogenic trypanosomes in the examined cattle. In Kenya, Ng'ayo *et al*. (2005) reported that PCR was 17.8 times more sensitive in detecing trypanosmes from small ruminants than microscopy. de Almeida *et al*. (1997) and Solano *et al*. (1999) reported that PCR was two times more sensitivite than microscopy in diagnosing trypanosome infected animals.

The higher sensitivity of PCR could be contributed to the fact that amplification of DNA by PCR permits the amplification of parasite levels far below that detectable by microscopic inspection (Masake *et al.*, 1997; Moser *et al.*, 1989a). When less than 25,000 parasites/ml were present in blood samples, the parasite is not detectable by microscopic diagnosis (Herbert and Lumsden, 1976). The higher sensitivity of PCR over microscopy permits the diagnosis of infection at stages before the parasitemia was observed using microscopy. Clausen *et al*. (1999) supported the high sensitivity of PCR by the findings that specific PCR products could be amplified from blood samples of experimentally infected animals collected on day one post infection; whereas trypanosomes were detected by microscopy four days post infection. In naturally infected animals, PCR identified 50% (Duvallet *et al.*, 1999) and 43.5% (Mugittu *et al.*, 2001) of microscopically negative animals to be infected. The obtained results and the published findings concluded that despite their simple application, parasitological techniques provide inaccurate and inadequate data, which shows the pitfalls of relying on these techniques for the control and treatment decision (Picozzi *et al*., 2002).

In the present work, one animal was found to be positive by microscopy for *T. vivax*, while, by testing different sample preparations using ITS-PCR, the results were negative for any species. This false negative result may be due to presence of PCR inhibitors in the blood sample or the contamination of the sample with exogenous DNA or DNAases that provoke the destruction of DNA (Desquesnes, 1997; Desquesnes and Davila, 2002; Solano *et al*., 2002). Other possible explanations of this result are that the primers used were not able to amplify the DNA from certain species or the sample was not prepared well leading to loss of the DNA (Masake *et al.*, 1997; Solano *et al.*, 1999).

3.6.1.3 Individual pathogenic species

The overall prevalence of different trypanosome species was determined by microscopy and PCR amplification in samples from 84 animals. The results indicated that the overall prevalence of *T. brucei* s.l., *T. congolense*, *T. vivax* and *T. theileri* in the 84 cattle examined by PCR was 13.1%, 7.1%, 8.3% and 53.6%, respectively. While the prevalence of *T. brucei* s.l., *T. congolense* and *T. vivax* in the examined cattle when determined by microscopy was 2.4%, 1.2% and 13.1%, respectively.

The overall prevalence of *T. brucei* s.l. in cattle examined by PCR and microscopy was 8.3% and 1.2%, respectively, with PCR seven times more sensitive than microscopy. This is in agreement with several studies that compared the use of microscopy and PCR amplification in detecting *T. brucei* s.l. Clausen *et al*. (1999) revealed that the prevalence of *T. brucei* s.l. in the examined cattle by microscopy and PCR was 5.3% and 9.9%, respectively.

The prevalence of *T. vivax* in cattle in the present work as determined by microscopy was 13.1% while by PCR was 8.3%. Noticing the type of species identified by PCR in the six samples positive for *T. vivax* using microscopy and missed by PCR, it was found that PCR amplified *T. theileri* in five samples and only one sample was identified as *T. brucei* s.l. The possible explanation of this condition is these six animals might have mixed infection of *T. brucei* s.l*/T. theileri*, and microscopy misclassified *T. brucei* s.l. as *T. vivax* (Delafosse *et al.*, 2006) and was unable to identifiy *T. theileri* due to low parasitemia.

Concerning *T. congolense*, the overall prevalence as determined by microscopy and PCR was 1.2% and 7.1%, respectively, with PCR seven times more sensitive. The results obtained from the same study area by McOdimba (2006) showed ten times more sensitivity of PCR in diagnosing *T. congolense*. This difference in the results might be due to different factors including, different primers used by the author targeting specific repetitive sequence for *T. congolense* Savannah with a higher copy number (5400), the use of buffy coat samples prepared from spinning down 10 ml blood and screening of eight FTA ®card discs from each sample. Another explanation could be the less sensitivity of the primers used in amplification of *T. vivax* DNA.

The overall prevalence of pathogenic trypanosomes in the current study was found to be 28.6% as determined by PCR. This finding was lower than that obtained by McOdimba (2006) who found that the prevalence of trypanosomes in the same districts when using PCR amplification was 44.3%. This difference might be attributed to that the author used primers specific for *T. congolense*, *T. brucei* s.l. and *T. vivax* that are more sensitive and specific for the aforementioned species due to amplification of higher copy number targets (Chapter V) and the screening of hogher volume of the sample.

Mixed infections were mainly diagnosed by PCR (8.3%) rather than microscopy (1.2%), indicating that PCR was 7.9 times more sensitive than microscopy in detecting mixed infections (χ^2=5.75 and $p<0.03$). This finding is due to the lower sensitivity of microscopy to differentiate between different species in mixed infections (Majiwa *et al.*, 1994; Moser *et al.*, 1989a) or mis-diagnosis by microscopy. The type of the identified mixed infections using PCR was 1.2% *T. brucei* s.l*/T. congolense*, 3.4% *T. brucei* s.l*/T. theileri* and 3.4% *T. vivax/T. theileri*. Microscopy identified only one mixed infection of *T. brucei* s.l*/T. vivax* combination; however, *T. brucei* s.l. in this animal was not identified using PCR. This could be due to the low sensitivity and specificity of ITS-PCR in amplifying *T. brucei* s.l. DNA (fully discussed in Chapter V). Solano *et al*. (1999) reported similar

findings of PCR diagnosis of four mixed infections which couldn't be detected by parasitological examination.

These results are in agreement with other results obtained by different authors in different studies to detect trypanosome species (Clausen *et al*., 1998; Kaare *et al*., 2006; Mugittu *et al*., 2001; Njiru *et al*., 2005; Waiswa *et al*., 2003). In a first trial of using PCR to detect cattle infected with trypanosomes in Zambia, Katakura *et al*. (1997) reported that by examining 100 blood samples applied on filter papers using specific primers for *T. brucei* s.l. and *T. congolense* Savannah, 29 animals were found to have mixed infections with both species. While, by examining thin smears from the same samples, two mixed infections with these two species were observed. These findings concluded that PCR technique is a sensitive diagnostic tool not only for trypanosome identification but also for the detection of mixed trypanosome infections.

3.6.2 Suitability of various sample preparations for the accurate diagnosis of trypanosome infected animals

In order to determine the suitable sample preparation for the accurate diagnosis of trypanosome genomic material by PCR amplification, the sensitivities of detecting trypanosome DNA in FTA®cards and DNA purified solutions were studied. The different sample preparations included whole blood, buffy coat and DNA applied on FTA®cards and extracted DNA solutions from whole blood and buffy coat. The detection of trypanosomes in the aforementioned materials was compared to a gold standard which was defined as positive PCR result from amplifying trypanosome DNA from FTA®cards and/or positive PCR result from amplifying trypanosome DNA in homogenous solutions.

The specially designed surface of an FTA®card allows the lysis of the cells upon application; however, there is no homogenous distribution of the entrapped DNA. Assuming that 100 µl sample contains only one trypanosome cell, positive PCR result requires the presence of the target DNA in the examined disc. Screening one disc is equivalent to 1.34 µl from the sample (Becker *et al.*, 2004), examining several discs represents an increase in the volume of the original sample screened. In the current study, 100 µl of blood was applied on FTA®cards and five discs were screened and they were equivalent to screening 6.7 µl of blood sample.

Buffy coat was obtained by spinning down 4 ml whole blood, and then 100 µl of the buffy coat was applied to FTA®cards. Assuming that the buffy coat is representative of the original blood sample, 1 µl buffy coat is equivalent to 40 µl blood. Examining five discs from the buffy coat applied on FTA®card is equivalent to examining 6.7 µl of buffy coat which in turn is equivalent to screening 268 µl from the original blood sample.

Extracting the DNA in solution ensures the homogenous distribution of the genomic material. DNA extraction from 300 µl whole blood was performed using GFX extraction kits. Assuming that the

blood sample to be subjected to DNA extraction contained one trypanosome, after sample lysis, the resulting solution becomes "contaminated" by the parasitic DNA with 200 copies per genome of the target. This contaimination is such that you would expect every 1 µl of DNA examined there after by PCR to be positive. Application of 100 µl of the extracted DNA solution on FTA®card should give the same result when screening only one disc because it should present a homogenous surface "contaminated" by the DNA of the single trypanosome. However, this was not found to be the case, although one disc from the same whole blood DNA extract (GFX) applied on FTA®cards should have contained 2.7 copies of the target, screening five discs (containing 13.4 copies of the target if one trypanosome cell was contained in the sample) failed to amplify the trypanosome DNA. This indicated that it was not easy to release the entrapped DNA from the FTA matrix; this could be due to the long time storage (four years) of the samples on FTA®cards (Becker *et al.*, 2004). Although detecting trypanosome DNA from homogenous extract (GFX) on FTA®cards was not as expected, but the senisitivty was 2.5 times more compared to the amplification of trypanosomes DNA from whole blood and buffy coat samples applied on FTA®cards, however this was not significant.

Based on the same assumption used to calculate the level of content of blood with parasitic material, let us imagine that we have 1 ml of blood contains 3.33 parasites (one parasite per 300 µl). A further assumption is that these parasites are contained with the buffy coat following centrifugation, if this buffy coat is then extracted and lysed it will be "contaminated" by these parasites at a factor of 3x greater than the whole blood DNA preparation. Interestingly, with analysing the buffy coat DNA extract we found this not to be the case.

In the case of extracting DNA from buffy coat, 1 ml of blood sample was spun down to collect 100 µl of buffy coat layer, so 1 µl of buffy coat is equivalent to 10 µl whole blood. Assuming that one trypanosome cell was included in the sample, after lysing the sample using the lysis buffer in the Qiagen extraction kits, each µl of the lysed sample contained two copies of the ITS target. After extraction, DNA was eluted in 200 µl eluting buffer, meaning that screening 1 µl from the extract should have been ampilified as it contained one copy of the target. According to the aforementioned hypothesis, it was expected to obtain the same positive results from the samples of DNA extract preparations either on FTA®cards or in homogenous DNA extract.

3.6.2.1 Diagnosis of any trypanosome species

The results in Table 3.9 indicated that the sensitivity of PCR to amplify any trypanosome DNA from 1 µl of DNA homogenous solution extracted from whole blood was significantly higher than amplifying 1 µl of DNA solution extracted from buffy coat (Q) by a factor of 1.4. This was expected because examining 1 µl from whole blood DNA extract should contain twice the copy number of the target contained in the same volume of buffy coat DNA extract.

These results are in disagreement with those obtained by Alhassan *et al*. (2007) who compared different sample preparations for the diagnosis of *Theileria equi* using PCR. The samples compared were blood applied on FTA®cards and extracted DNA using Qiagen kits and phenole extraction using different parasite concentrations from 10-10^{-5}. The results revealed minimal variation in the limit of detection between the used sample preparation methods. The authors concluded that *Theileria equi* can be detected directly from whole blood samples using PCR amplification from single FTA®card discs. This could be attributed to the use of more FTA discs (the authors mentioned only the use of multiple 3 mm) which increased the probability of obtaining the target of interest in the screened discs. Another explanation would be the use of fresh samples applied on FTA®cards which in turn made it easier to wash out the blood impurities from the examined discs and release the DNA from the matrix during the PCR cycles. However, in the current study; samples were preserved in FTA®cards four years prior to screening which made it difficult to remove the impurities from the sample. This conclusion was supported by Becker *et al*. (2004) who reported that long term storage (more than nine months) could prevent the complete removal of blood from the discs by washing. Nevertheless, washing the discs for longer time may lead to the removal of the DNA from the card matrix or inhibition of the PCR reaction

By calculating the Kappa values for the degree of agreement between the detection of trypanosomes in different sample preparations and the gold standard, it was found that there was a very good agreement between the detection of the trypanosome DNA using whole blood DNA extract (Kappa value= 0.8) when compared with the gold standard. There was less agreement between the gold standard and detecting the trypanosomes in buffy coat DNA extract (Kappa value=0.5). However, no significant agreement was found between the gold standard and the ampilification of trypanosome DNA from different materials applied on FTA®cards (Table 3.10). There was no significant difference between the amplification of the trypanosome genomic material from FTA discs contained blood and buffy coat, although the equivalent examined volume of screened blood was 6.7 µl and 268 µl from whole blood and buffy coat preparations, respectively. This is not in agreement with the findings previously reported by Woo (1971), Murray (1977) and Picozzi *et al*. (2002) who suggested that the detection of trypanosomes in samples prepared from buffy coat was significantly higher than whole blood due to the concentration of trypanosome cells in the buffy coat layer.

3.6.2.2 Diagnosis of pathogenic trypanosome species

Excluding the non-pathogenic *T. theileri* from the analysis, the results show that a highly significant agreement between both DNA extract from blood (GFX) and buffy coat (Q) was observed when compared to the gold standard. This was expected due to the assumed existence of at least one copy of the target sequence when using 1 µl from the homogenous solution. Application of DNA extracted from whole blood (GFX) on FTA®cards and examining 5 discs resulted in 18.2% sensitivity in amplifying the target. However, examining 1 µl from the same extract resulted directly in 4.3 times more sensitivity, although this was not significant.

3.6.3 Proportion and species of trypanosomes detected by ITS-PCR in the examined materials

Among the 84 animals examined, eight were found to harbour mixed infection by PCR of DNA extract. However, application of five separate PCR reactions on samples applied on FTA®cards failed to detect such mixed infections. Therefore, blood and buffy coat applied on FTA®cards of the animals with mixed infection were further screened by increasing the number of examined discs from each material to 30. The volume of blood equivalent to screening 30 discs from whole blood applied on FTA®card was 40.2 µl, whereas 30 discs from buffy coat samples on FTA®card were equivalent to 1608 µl of the original blood sample. It was expected to detect more positive discs using PCR; however, this was not the case. The results of the 30 separate discs from both blood and buffy coat on FTA®card discs revealed that the reactions failed to detect the mixed infection.

These findings could be due to the un-even distribution of the DNA on the FTA®card matrix due to the localised immobilization of the DNA when come in contact with the matrix after cell lysis. However, the screening of the 30 discs should have picked the DNA due to increased volume of examined sample, this supports the argument that the DNA release from the discs seemed to be difficult. Moreover, the long storage of the samples used in the current study might be another factor in the difficult release of the entrapped DNA from the FTA matrix (Becker *et al.*, 2004). Cox (2007) screened between 92-114 discs (~123.3 µl – 152.8 µl) containing whole blood and 85.7% of the samples were positive for trypanosomes. Mixed infection was detected in 60% of the samples, with *T. theileri* included in all types of the mixed infections. This disagreement with the current results could be due to the higher volume of blood sample examined and the ability to release DNA from the matrix due to screening the samples shortly after collection. The low prevalence of mixed infections compared with single infections with the predominance of *T. theileri* in the current study, could be due to the reduced ability of other trypanosome species to be established in the presence of *T. theileri* giving a probability that there may be competition occurring between *T. theileri* and other trypanosomes in the host (Cox, 2007). Another explanation to the low prevalence of mixed infection is the presence of defence mechanism either within the vector or vertebrate host.

In conclusion, the work presented aimed to evaluate FTA®cards for sample collection to be diagnosed for trypanosome using PCR. The main drawback encountered with FTA®card matrix is the uneven localised distribution of the DNA when in contact with the matrix after cell lysis. This requires the presence of the DNA in the examined disc to be amplified and diagnosed with PCR. However, lysing the cells to release the genomic material and extracting the DNA in homogenous solution ensures the presence of the target in as little as 1 µl of the homogenous extract. The main obstacles of the DNA extraction approach are time consumption, requirement of certain equipments and the highly economic cost (Desquesnes and Tresse, 1996; Devost and Choy, 2000). In order to overcome the

drawbacks of DNA extraction and to obtain a homogenous solution of DNA using FTA®cards, the forthcoming work aimed to optimise sample collection on FTA®cards.

4 Chapter four

Improvement of blood sample preparation from FTA®cards for trypanosome diagnosis

4.1 Introduction

The results obtained in Chapter III, showed that the detection of trypanosome DNA from whole blood and buffy coat extracts was significantly more sensitive than from whole blood or buffy coat applied directly on FTA®cards (χ^2=56.9, p<0.001). This can be interpreted as either the inability to access parasitic DNA on the card matrix or the uneven distribution of the genomic material across the surface of the card.

In order to improve the accuracy of sample screening, trials were undertaken to modify the original protocol for the preparation of blood prior to application onto the FTA®cards by the introduction of a lysis step. Cell lysis can be caused by excessive osmosis or movement of water towards the inside of a cell. Eukaryotic cell membranes can not withstand the osmotic pressure of the water inside, leading to explosion of the cell and release of its contents including DNA (Christensen and Hoffmann, 1992; Wang *et al.*, 2008). Lysis promotes the release of the DNA into a homogenous solution consisting of genetic material from both the host as well as any infectious agent. The probability of detecting the parasitic DNA in the examined material is therefore increased with a more even distribution of the parasitic DNA over the surface of the FTA matrix. Lysis, if successful, would therefore avoid time consuming, laborious and expensive DNA extraction in the field.

Different studies have been conducted to compare between different methods to extract DNA using different commercial DNA extraction kits and the use of Chelex®100 to elute DNA from the same samples. Chelex®100 is a chelating resin that has a high affinity for polyvalent metal ions, moreover, the alkalinity (pH 10-11) and the exposure to temperature result in the disruption of the cell membrane and denaturation of DNA into its component single strands facilitating PCR amplification (Sweet *et al.*, 1996; Walsh *et al.*, 1991).

For example, in the field of forensic medicine, a study to compare between five different methods to extract DNA from decomposed tissues including phenol-chloroform extraction, silica based, InstaGene Matrix™ (Bio Test), glass fibre filter and the Chelex®100 based method, was conducted by Hoff-Olsen *et al*. (1999). The aim of the study was to compare the time required to perform the extraction, the cost of the method and the quality of the electrophoretic product by each method. The Chelex®100 based extraction was proven to be the most rapid and least expensive method (less than 0.01$ per extraction), but failed to produce electrophoretic product especially from degraded tissue samples. Another evaluation of DNA extraction using Chelex®100 was done by performing a comparison of extracting DNA from hair using Chelex®100 compared to QIAamp® Mini Kit method and ISOHAIR® method (Suenaga and Nakamura, 2005). The authors recommended the use of Chelex®100 because of its simplicity and low cost when compared with the other methods used. Moreover, PCR of DNA extracted using Chelex®100 was shown to be less affected by inhibitors and at the same time gave a reasonable quantity of extracted DNA (120-140 ng). Also, an increase in the signal strength from PCR amplification was noticed using Chelex®100 (Singer-Sam *et al.*, 1989). The

benefits from using Chelex®100 to extract DNA from blood has been shown to be six-fold increase in extraction efficiency, the use of non-organic solvents and the expenditure of less analyst time, effort and cost (Jung *et al.*, 1991). This is in contrary to the use of conventional methods of DNA extraction that usually require large volume of blood, the use of health hazard organic solvents and the time and cost consuming (Loparev *et al.*, 1991).

Chelex®100 has been used to extract DNA from different samples that are important from the forensic point of view, including, blood (Jung *et al.*, 1991), decomposed tissues (Hoff-Olsen *et al.*, 1999), hair (Suenaga and Nakamura, 2005), waxed tissues (Sepp *et al.*, 1994), saliva (Sweet *et al.*, 1996), incinerated teeth (Tsuchimochi *et al.*, 2002), semen (Crouse *et al.*, 1993) and vaginal swabs (Iwasa *et al.*, 2003). Moreover, it has been documented that Chelex®100 has been used to extract DNA in fields other than forensic medicine such as, plant diseases. Chunwongse *et al*. (1993) extracted DNA from half-seeds using Chelex®100, the produced amplicon was comparable to those obtained using proteinase K treatment but with less time and cost.

In the virology field, Chelex®100 was used to extract human immunodeficiency virus (HIV) from blood samples in a study conducted by Vignoli *et al*. (1995). The increase in the yield of PCR products was attributed to many factors, firstly, the protective effect of Chelex®100 against DNA degradation. Secondly, the less chance of sample contamination and finally the removal of most PCR inhibitors through chelation with the resin. Moreover, the authors argued that the eluate extracted by Chelex®100 can be stored at -20ºC and used for PCR several months later (Vignoli *et al.*, 1995).

In parasites isolation and identification, Chelex®100 was used to extract the genomic material of *Theileria parva* (Geysen *et al.*, 1999; Polski *et al.*, 1998) and *Plasmodium falciparum* (Wooden *et al.*, 1993) from blood samples. Concerning trypanosome DNA extraction from different samples, Chelex®100 was used to elute trypanosome genomic material from blood (Boid *et al.*, 1999; de Almeida *et al.*, 1997; de Almeida *et al.*, 1998b; Jamonneau *et al.*, 2004; Katakura *et al.*, 1997; Solano *et al.*, 2002), buffy coat (de Almeida *et al.*, 1998a; Solano *et al.*, 2002; Solano *et al.*, 1999) and CSF (Truc *et al.*, 1999) to follow up the treatment programme and to determine the stage of the disease using PCR.

Various studies have proven that prophyrin compounds that are present in the heme component of the red blood cells are one of the major PCR inhibitory components of blood. These compounds have been shown to inhibit the activity of the thermo-stable DNA polymerases used in the PCR reaction (Akane *et al.*, 1994; Cox, 2007; de Franchis *et al.*, 1988; Loparev *et al.*, 1991; Wooden *et al.*, 1993). The higher suitability of hot eluates was attributed to the removal of the PCR inhibitors and release of the bound DNA to the filter paper matrix into the eluate by boiling (Boid *et al.*, 1999). If just water is used, the elaute will not remain stable due to the presence of inhibitors and also due to the easiness of DNA degeneration. However, DNA extraction from blood using Chelex®100 is less likely to have PCR inhibitors because Chelex®100 helps in the removal of such inhibitors by binding to the

Chelex®100 bead matrix itself (Walsh *et al.*, 1991). From the previous studies, it is clear that the use of Chelex®100 is effective in extracting highly pure template DNA from blood samples that are notorious for the carryover of PCR inhibitors (Polski *et al.*, 1998). Because it seems to overcome the problems associated with inhibitory effects of the blood components such as heme, lactoferrin, IgG and non-target DNA (Cox, 2007; de Almeida *et al.*, 1997). Moreover, Chelex®100 protects DNA from the effect of high temperature that causes degradation of DNA by chelating metal ions which might act as catalysts in the breakdown of DNA at high temperature in low ionic strength solutions (Singer-Sam *et al.*, 1989).

In contrast to the previous studies, Becker *et al.* (2004) reported that the presence of PCR inhibitors cannot be completely ruled out after Chelex®100 extraction. The authors observed low reaction efficiency values when Chelex®100 was used for eluting DNA from blood samples applied on FTA®cards for quantifying trypanosomes DNA using quantitative PCR. They argued that the low efficiency values indicated that Chelex®100 extraction does not remove all inhibitory substances from blood samples, however, the template DNA obtained by this method leads to quantitative results. Another study was conducted by Reithinger *et al.* (2000) to detect *leishmania* species in dog blood using PCR in Chelex®100 extracted samples. The authors reported that none of the Chelex®100 extracted samples amplified the target DNA. They attributed this failure to the presence of PCR inhibitors that were not removed using Chelex®100.

4.2 Objectives

The aim of this study is to improve blood sample preparation from FTA®cards for trypanosome diagnosis. A very sensitive PCR protocol directed against a repetitive sequence existing in the genome at a copy number of more than 10,000 (Moser *et al.*, 1989a; Sloof *et al.*, 1983a) was chosen. This aim was achieved by:

1- Application of experimental lysis trials using serial dilutions of *T. brucei* s.l. to end with concentration of less than one parasite/100 µl applied on FTA®cards thereby releasing the trypanosome genomic material in a homogenous solution to increase the probability of detecting trypanosome DNA on FTA®cards.

2- The field application of the lysis approach was carried out on two scales; a preliminary small scale lysis trial comparing the lysed samples with the conventional whole blood samples applied on FTA®cards, and the large scale lysis trial was applied on a larger number of samples and compared to elution of DNA using Chelex®100 from the whole blood samples collected on FTA®cards and *in situ* DNA extraction.

4.3 Material and methods

4.3.1 Experimental lysis of blood samples

4.3.1.1 Preparation of the procyclic insect forms

Procyclic trypanosomes (insect form trypanosomes) were obtained by infecting *Glossina morsitans morsitans* with bloodstream form trypanosomes (isolate Buteba 135 originally isolated from a cow in Buteba village, Uganda in 1990 (Dale *et al.*, 1995)) and dissecting mid-guts from tsetse 10 days post-infection (Maudlin, 1996). Before dissection, tsetse flies were sterilised with 5% sodium hypochlorite (VWR, UK) followed by rinsing in sterile saline. Trypanosome infected mid-guts were then placed in a 24 well plate (SLS, UK) in 1 ml Cunningham's medium (Invitrogen, UK) containing 20% foetal calf serum (Invitrogen, UK) and 120 µg/ml gentamicin (Sigma, UK). Cunningham's media was made to the recipe of Cunningham (1977) by Invitrogen (UK).

Once the procyclic culture was established, it was transferred to 25 cm^3 culture flask (SLS, UK) and diluted every other day 1:10 in fresh Cunningham's medium containing 20% foetal calf serum and 20 µg/ml gentamicin. One ml of procyclics was centrifuged at 4000 rpm for three minutes, the supernatant was discarded and the pellet remaining was re-suspended again in a fresh Cunningham's media. The dilutions were examined under the microscope (magnification=x400) and the dilution that had one parasite per 20 microscopic fields was equivalent to $2.5x10^5$ parasites/ml (Herbert and Lumsden, 1976). Serial dilutions (from $2.5x10^5$- 2.5 parasites/ml) were then made in Cunningham's media to obtain less than one parasite/100 µl for application on FTA®cards.. For controls, 100 µl from each dilution was taken out and applied to FTA®cards (Table 4.1).

Table 4.1: Number of parasites and the equivalence in the examined discs (controls)

Number per ml	Number per 100 µl	Number per one disc	Chance of isolating a parasite in one disc
$2.5x10^5$	$2.5x10^4$	335	100%
$2.5x10^4$	$2.5x10^3$	33.5	100%
$2.5x10^3$	$2.5x10^2$	3.35	100%
$2.5x10^2$	25	Less than one (0.335)	33%
$2.5x10^1$	2.5	Less than one (0.0335)	3%
2.5	0.25	Less than one (0.00335)	0.3%

4.3.1.2 Preparation of the infected blood dilutions

TO (BKl:BLW) mice (B and K Ltd, UK) were immunosuppressed by intra-peritoneal injection of 80 mg/kg cyclophosphamide (Pharmica and Upjohn Ltd) and infected from a thawed *T. b. brucei* stabilate (Buteba 135) 24 hours later by intra-peritoneal injection (Welburn and Maudlin, 1987). Regular checks were made by microscopical examination of blood. When the parasitaemia reached

between 10^8 and 10^9 parasites/ml; the blood from the mice was collected by cardiac puncture under terminal anaesthesia.

Dilutions of the infected blood using non infected mouse blood were examined under the microscope (magnification= x400) in order to estimate the concentration of parasites per ml. The dilution that had one parasite per 20 microscopic fields was equivalent to $2.5x10^5$ parasites/ml (Herbert and Lumsden, 1976). Serial dilutions (from $2.5x10^5$- 2.5 parasites/ml) were then made to obtain less than one parasite/100 µl for application on FTA®cards. This aimed to determine the sensitivity of the lysis approach in releasing sufficient DNA from less than one trypanosome cell into a homogenous solution before application onto the FTA matrix. Before the addition of the lysis agent to the prepared dilutions, 100 µl of each dilution was applied on FTA®cards as a control (Table 4.1).

4.3.1.3 Preparation of the lysis agents

The lysis agents used were H_2O, 1% (w/v) SDS (Sodium Dodecyl Sulfate, Sigma) in distilled water and hypotonic PBS (Phosphate Buffer Saline tablets, Sigma). PBS was prepared by dissolving one tablet in 200 ml water to obtain: 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride with pH 7.4.

4.3.1.4 Lysis trials

4.3.1.4.1 First experimental lysis trial

The first trial was performed using procyclic forms to investigate the efficiency of lysing the cells using different lysing agents. Serial dilutions from procyclic forms starting from a concentration of $2.5x10^5$ were made in Cunningham's media as a diluent. After preparing the serial dilutions, to micro-centrifuge tubes, 50 µl of each diluent containing procyclic forms and 50 µl of each lysis agent (H_2O, PBS and 1% SDS) were added. The tubes were incubated at room temperature for three hours and then 100 µl was applied on FTA®cards and left for one hour to dry (Table, 4.2). The controls used in this trial were 100 µl taken from each serial dilution before adding the lysis agents and applied on FTA®cards (Table 4.1).

Table 4.2: Number of parasites per ml and the equivalence in the examined discs after lysis

Number per ml	Number per 50 µl	Number of parasites applied to FTA®cards*	Estimated copy number of the target in one disc
$2.5x10^5$	$1.25x10^4$	$1.25x10^4$	$1.68x10^6$
$2.5x10^4$	$1.25x10^3$	$1.25x10^3$	$1.68x10^5$
$2.5x10^3$	$1.25x10^2$	$1.25x10^2$	$1.68x10^4$
$2.5x10^2$	12.5	12.5	$1.68x10^3$
$2.5x10^1$	1.25	1.25	$1.68x10^2$
2.5	Less than one (0.125)	Less than one (0.125)	16.8

***This indicates the number of parasites from which DNA content was released into the homogenous solution after lysis**

Table 4.2 shows the number of parasites/ml in each dilution and the number of parasites after lysis from which DNA was released into solution. Relating the DNA released from the parasite number in

each dilution to the copy number of the PCR target, the table shows the copy number of the target in each examined disc. For instance, after lysing the sample containing $2.5x10^3$ parasites/ml; 100 µl from the lysed sample contained DNA from $1.25x10^2$ released in homogenous solution. Each examined disc is equivalent to 1.34 µl, therefore one disc would contain DNA from 1.7 (~2) parasites and the copy number of the target in this disc was $1.68x10^4$ copies which in theory should be enough for amplification.

4.3.1.4.2 Second experimental lysis trial

The second lysis trial was carried out using procyclic forms diluted in Cunningham's media (the same protocol as the first trial) with the addition of cow's DNA (natural host DNA) to match the natural conditions where both the parasitic and host DNA co-exist in the sample. It was assumed that 50 µl of bovine blood would contain 1.6 µg DNA (Qiagen extraction kits, 2007). The DNA was extracted from blood using DNeasy®Tissue Extraction kits (Qiagen) (100 µl of anticoagulated blood was added in 1.5 ml tube containing 20 µl proteinase K, the volume was then adjusted to 220 µl using PBS and the extraction procedure was then completed as previously detailed in Chapter III, section 3.4.3.2). After lysis took place, 5 µl of extracted DNA was added to each tube (each 1 µl of extraction buffer contains 0.2-0.4 µg DNA). The control used in this trial was 100 µl taken from each serial dilution containing host DNA before adding the lysis agents and applied on FTA®cards (Table 4.1).

4.3.1.4.3 Third experimental lysis trial

In this trial, lysis of bloodform trypanosomes from vertebrate host was investigated. Trypanosome cells were collected from an experimentally infected mouse. Serial dilutions were made from a starting concentration of $2.5x10^5$ using uninfected mouse blood as the diluent. After preparing the serial dilutions, in micro-centrifuge tubes, 50 µl of each diluent containing infected blood forms and 50 µl of each lysis agent (H_2O, PBS and 1% SDS) were added. The tubes were incubated at room temperature for three hours (to match the conditions in the field and to give a chance for lysis to occur). Afterwards, 100 µl was applied on FTA®cards and left for one hour to dry (Table, 4.2). The controls used in this trial were 100 µl taken from each serial dilution before addition of the lysis agents and applied on FTA®cards.

4.3.1.5 TBR-PCR

TBR-PCR is a species-specific reaction that amplifies 173 bp product from a repetitive satellite sequence of 177 bp size (Moser *et al.*, 1989a). The copy number of the satellite sequence was reported to be 10,000 copies/genome (Sloof *et al.*, 1983); which lead to the appearance of a double band at 346 bp due to the repetitiveness of the sequence (Figure 4.1). The primers sequence used were TBR1: 5'-CGA ATG AAT ATT AAA CAA TGC GCA GT-3' and TBR2: 5'-AGA ACC ATT TAT TAG CTT TGT TGC-3' (Moser *et al.*, 1989a).

The reaction volume used was 25 µl and contained NH_4 Buffer (Bioline, London, UK) that is formed of 16.0 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH8.8 at 25ºC) and 0.01% Tween 20. Moreover, 0.4 µM of each primer, 1.5 mM Mg^{2+}, 200 µM of each of the four-deoxynucleoside triphosphates (dNTP) and one Unit of BIOTAQ RED DNA Polymersase (Bioline) were added. One positive control (genomic DNA) and one negative control were run with each PCR.

The reaction conditions were as follows: 1 cycle of 95ºC for seven minutes followed by 30 cycles of 94ºC for one minute, 55 ºC for one minute and 72 ºC for 30 seconds. The reaction was carried out in a DNA Engine DYADTM Peltier Thermal Cycler. Figure 4.1 shows a gel picture of TBR-PCR positive results.

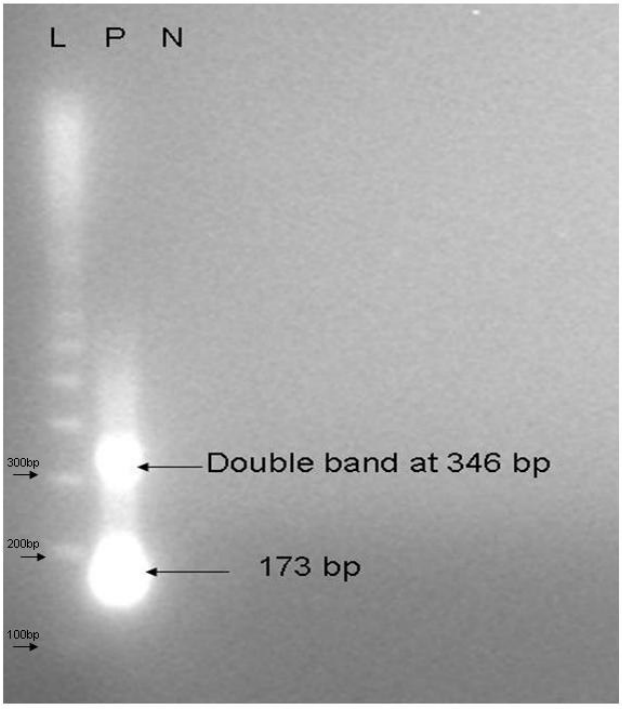


Figure 4.1: Example of TBR-PCR results in 1% agarose showing the product at 173 bp and the double band that results from the repetitiveness of the target at 346 bp (L: 100 bp ladder, P: positive control, N: negative control)

4.3.2 In field application of lysis

In field application of the blood lysis approach was carried out over two stages; the first was a preliminary lysis study (June, 2006) in which a total of 80 blood samples were collected from cattle in Uganda. The second in field application was done using a larger sample set (300) and these were

collected in Uganda, October, 2006. Figure 4.2 shows a map of Uganda with the sampling sites in both the preliminary and large in field lysis application.

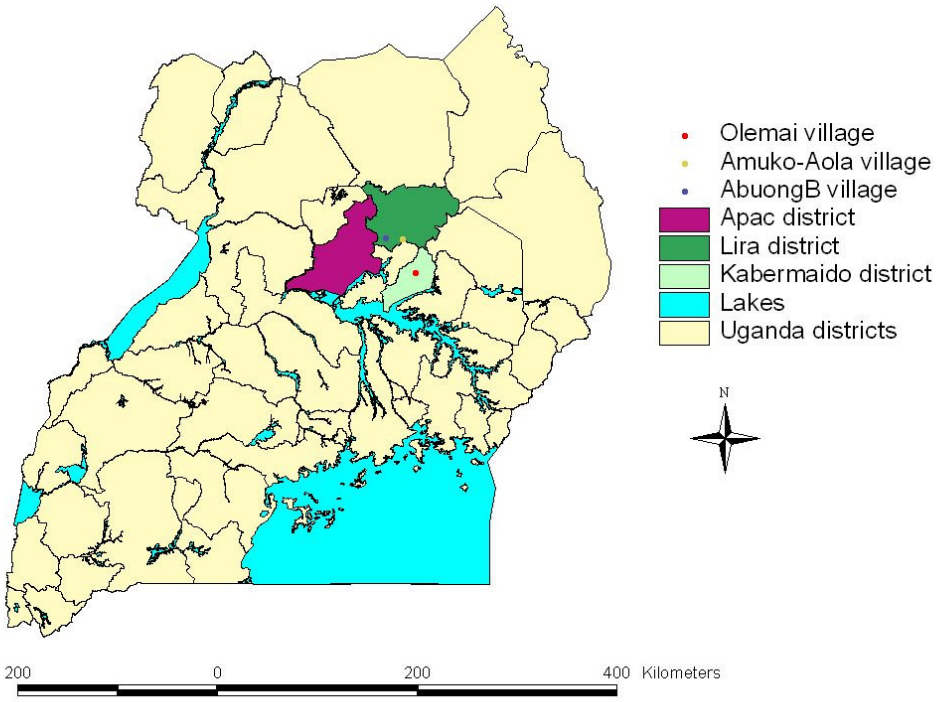


Figure 4.2: Uganda map showing the in field lysis sampling sites

4.3.2.1 Preliminary in field lysis application

4.3.2.1.1 Study area

Samples were collected during a survey in Uganda to estimate the prevalence of trypanosomes in a newly affected area with *T. b. rhodesiense*, before the initiation of a mass treatment campaign to stop the further spread of the parasite (Stamp Out Sleeping sickness campaign, Chapter VI). One site (Olemai village, Lwala parish, Kabermaido district) was randomly chosen by the SOS group to apply the lyses protocol on blood samples collected from the 80 cattle assembled in the chosen site. Figure 4.2 shows the selected site (Olemai village), Eastern Uganda.

4.3.2.1.2 Sample collection

Collection of whole blood samples

After lancing the animal ear vein (Figure, 4.3), blood was collected into two heparinised capillary tubes of 50 µl capacity, each. After collection of whole blood samples, they were applied on FTA®cards. While pipetting the sample, the capillary tube tip was held slightly above the surface of

the FTA matrix and the blood was dispensed in a concentric circle pattern within the printed card circle (Figure, 4.4). The card was then allowed to air dry for about one hour at room temperature then sealed in envelopes containing desiccant to be transferred for analysis.

Figure 4.3: Bleeding of the ear vein for sample collection

Figure 4.4: Blood samples applied on FTA®cards

Collection of lysed blood samples

One heparinised capillary tube of blood (50 µl) was collected from the ear vein of the animal. The content of the capillary tube was allowed to flow into an Eppendorf tube containing 50 µl of distilled water. The tubes were sealed and lysis was allowed to take place during three hours incubation at ambient temperature. After that, 100 µl of the lysed solution was removed and applied on FTA®cards and then the cards were allowed to dry before storage.

4.3.2.1.3 Sample processing

Preparation of the samples from FTA®cards

In this study, 10 discs from both whole blood and lysed blood applied on FTA®cards were examined by separate PCR (each disc examined with a separate PCR). The protocol for washing and preparing the discs for PCR were fully described in Chapter III (section 3.4.4).

TBR-PCR

The primer sequences, reaction conditions and product size are fully described in section 4.3.1.5 in this chapter.

4.3.2.2 Large scale field lysis application

4.3.2.2.1 Study area

In this study, 300 animals were sampled from three villages during baseline sampling of the Stamp Out Sleeping sickness campaign (Chapter VI) in Apac and Lira districts, Uganda (Figure, 4.2). Agwea village (Apac district) is not shown in the map due to inability to obtain the longitude and latitude data to include the village in the map. The choice of 300 samples was based on the availability of DNA extraction kits enough for only this number of samples. The sites of sample selection were selected according to the availability of at least 100 cattle in the visited sites; from each site 100 cattle were sampled.

4.3.2.2.2 Sample collection

Three sample preparations were collected (whole blood, lysed blood and DNA extract) to compare between the detection of trypanosomes using PCR in those preparations.

Collection of whole and lysed blood samples

Collection of the whole and lysed blood samples on FTA®cards was done as previously mentioned during the preliminary in field application (section 4.3.2.1.3).

In situ DNA extraction

For *in situ* DNA extraction, blood was collected into capillary tube containing EDTA to prevent coagulation of the blood. The tube was inserted into a collection tube for storage of the blood (MiniCollect, greiner bio-one) until the extraction protocol was processed.

4.3.2.2.3 Sample processing

Whole and lysed blood samples collected on FTA®cards

Ten discs from both whole blood and lysed blood applied on FTA®cards were examined by separate PCR (each disc examined with a separate PCR). The protocol for washing and preparing the discs for PCR were fully described in Chapter III (section 3.4.4).

Another 10 discs from each sample collected on FTA®cards (whole blood and lysed blood) were cut and placed into one micro-centrifuge tube. After washing and drying the discs (Chapter III, section 3.4.4), DNA was eluted by boiling the discs for 30 minutes at 90ºC in 60 µl of 5% (w/v) aqueous suspension of Chelex 100® resin (sodium form, 50-100 dry mesh, Sigma) (Becker *et al.*, 2004). For PCR, 5 µl of the eluate was added to 20 µl of the PCR master mix.

In situ DNA extraction

In situ DNA extraction was done using ChargeSwitch® gDNA kit. The principle of this extraction method is the use of magnetic beads. At low pH, the magnetic beads have a positive charge that binds the negatively charged nucleic acid backbone of DNA. Proteins and other contaminants do not bind and are removed by washing. For the elution of the bound DNA, the charge of the magnetic beads was neutralised by raising the pH to 8.5 using a low salt elution buffer. The purified DNA was released into the elution buffer; the yield from 50 µl of blood was up to 2 µg. For PCR, 1 µl from the extract was used as the template for the TBR-PCR reaction.

Procedure

DNA binding

To extract DNA from 50 µl whole blood in EDTA, 1ml lysis buffer (L10) and 20 µl of the magnetic beads were added to the sample. The sample was mixed by pipetting up and down gently five times. The tubes were incubated at room temperature for one minute before being placed on MagnaRack™ until the beads had formed a tight pellet. The supernatant was then discarded without removing the tubes from the magnet.

Proteinase K digestion

For each sample, 100 µl purification buffer (N5), 1ml lysis buffer (L11) and 5µl proteinase K were added after the tubes were removed from the MagnaRack™. The samples were mixed and incubated at room temperature for 5 minutes. After the tubes being placed in the rack for one minute, the supernatant was removed carefully from the tubes and discarded.

Washing of DNA

One ml wash buffer (W12) was added to the tubes after removing them from the MagnaRack™. The samples were mixed and placed in the rack for one minute; the supernatant was removed carefully from the tubes and discarded. This washing step was then repeated once more.

Eluting DNA

After removing the tubes from the rack, 100 µl Elution buffer (E5) was added. The samples were mixed by pipetting up and down 15-30 times. The tubes were incubated at room temperature for 10 minutes, and then placed on the rack for 2 minutes until the magnetic beads formed a tight pellet. Without removing the tubes from the MagnaRack™, the supernatant containing the DNA was transferred to a sterile tube.

4.3.2.3 Statistics

Sensitivity, negative predictive value and Kappa value were calculated as previously described in Chapter III (section, 3.4.8).

4.4 Results

4.4.1 Experimental lysis of blood samples

Lysis trials were made in three approaches using procyclics and bloodforms of trypanosomes. The first and second trials were performed using procyclics with the absence and presence of the host DNA, while the third trial was conducted in a vertebrate host to create a natural condition to asses the approach.

4.4.1.1 First experimental lysis trial

Procyclics were used to investigate the possibility of lysing the cells to obtain a more homogenous distribution of the trypanosome genomic material on the card matrix using different lysing agents. Five discs from each control (serial dilution of procyclics using Cunningham's media applied on FTA®cards) were examined using TBR-PCR on each disc. The same number of discs containing serial dilution of procyclic forms after lysis with the different lysis agents (H_2O, PBS and 1% SDS) were also examined by separate TBR-PCR reaction on each disc. Table 4.3 shows the number of discs reacting positive using TBR-PCR in the controls and the lysed procyclic forms.

Table 4.3: PCR results for the first experimental lysis trial (n=5 discs)

Lysis agent	Number of parasites/ml					
	$2.5x10^5$	$2.5x10^4$	$2.5x10^3$	$2.5x10^2$	25	2.5
Control (no lysis)	5	5	5	4	0	0
H_2O	5	5	5	4	5	5
PBS (Hypotonic)	5	5	4	5	5	5
1% SDS	5	5	5	5	5	5

The results in Table 4.3 show that with the higher procyclic concentrations in the controls ($2.5x10^5$-$2.5x10^2$), all the examined discs were positive for *T. brucei* s.l. However, at lower concentrations (25 and 2.5 parasites/ml) none of the examined discs contained the trypanosome DNA.

Although the lysed samples were half the concentration of the controls after the addition of the lysing agent, TBR-PCR was able to amplifiy the DNA from all the concentrations.

In this trial, no natural host DNA was present; these conditions therefore do not reflect the natural conditions where the host DNA would also been present. In order to match the natural conditions, another trial by using procyclics was carried out with the addition of natural host DNA (cow).

4.4.1.2 Second experimental lysis trial

In natural conditions, host DNA exists with the parasitic genomic material in the same sample. In order to match the natural conditions, this trial was made by adding extracted host DNA to find out if there was an effect of the host DNA on the detection of the parasitic genomic material using PCR amplification. The same protocol was used as described for the first trial with the addition of extracted host DNA (bovine) to the controls and to the lysed samples after the lysis step took place. Five discs from each control (serial dilutions applied on FTA®cards) were examined using TBR-PCR on each disc. The same number of discs containing serial dilution of procyclic forms after lysis with the different lysis agents (H_2O, PBS and 1% SDS) were also examined by separate TBR-PCR reaction on each disc. Table 4.4 shows the number of discs reacting positive using TBR-PCR in the controls and the lysed procyclic forms.

Table 4.4: PCR results for the second experimental lysis trial (n=5 discs)

Lysis agent	Number of parasites/ml					
	$2.5x10^5$	$2.5x10^4$	$2.5x10^3$	$2.5x10^2$	25	2.5
Control (no lysis)	5	5	3	3	0	0
H_2O	5	5	3	1	3	2
PBS (Hypotonic)	5	5	5	3	3	2
1% SDS	5	5	4	5	2	0

The results in Table 4.4 show that with the controls, TBR-PCR detected trypanosome DNA in the four high parasite concentrations. However, at the two low concentrations, none of the discs was positive by PCR. After application of cell lysis with different lysis agents (H_2O, PBS and 1% SDS), the PCR reaction detected the trypanosome DNA in all concentrations including the lower ones except for cells lysed using 1% SDS (2.5 parasites/ml). These results suggest the masking of the parasitic DNA with the host genomic material. This assumption was further investigated by lysing bloodforms using a vertebrate host to match natural field conditions.

4.4.1.3 Third experimental lysis trial

In this trial, an experimental infection of a vertebrate host (mice) was conducted to apply the lysis approach on bloodform cells to investigate the existence of a masking effect of the parasitic DNA with the host genomic material. Five discs from each control (serial dilution of bloodforms in mouse blood applied on FTA®cards) were examined using TBR-PCR on each disc. The same number of discs containing serial dilution of bloodforms after lysis with the different lysis agents (H_2O, PBS and 1% SDS) were also examined by separate TBR-PCR reaction on each disc. Table 4.5 shows the number of discs reacting positive using TBR-PCR in the controls and the lysed bloodforms.

Table 4.5: Positive discs with TBR-PCR from the first experimental lysis trial (n=5 discs)

Lysis agent	Number of parasites/ml					
	$2.5x10^5$	$2.5x10^4$	$2.5x10^3$	$2.5x10^2$	25	2.5
Control (no lysis)	4	4	4	4	0	0
H_2O	4	5	5	2	3	2
PBS (Hypotonic)	5	5	5	4	0	1
1% SDS	4	5	5	2	3	2

This trial was performed to apply the lysis protocol in natural infection conditions using bloodforms from a vertebrate host. The results show that *T. brucei* s.l. DNA was amplified in the higher parasite concentrations before lysing the cells; while none of the lower concentrations were found to be positive. After lysing the cells, trypanosome DNA was detected in all the concentration range except in case of using PBS hypotonic solution for lysis.

4.4.2 In field application of lysis

4.4.2.1 Preliminary in field lysis application

4.4.2.1.1 Overall results of TBR-PCR from all examined discs

A total of 80 cattle samples were collected, 10 discs from whole blood and another 10 discs from lysed blood were examined using TBR-PCR separately on each disc. The sample was considered positive when at least one disc reacted positive in the reaction, the results of TBR-PCR are summarised in Table 4.6.

Table 4.6: TBR-PCR results for 80 samples from whole and lysed blood

	Whole blood positive	Whole blood negative	Total
Lysed blood positive	1	15	16
Lysed blood negative	0	64	64
Total	1	79	80

The results in Table 4.6 show the overall PCR results from examining 10 discs containing whole blood and 10 discs containing lysed blood for the presence of *T. brucei* s.l. infections. It was observed that 16 animals were infected with trypanosomes when the lysis approach was used prior to application on FTA®cards. Only one animal was found to be infected with *T. brucei* s.l. by examining the un-lysed whole blood discs. The sensitivity of diagnosing the samples for *T. brucei* s.l. infection in whole blood discs was 6.3% compared to the amplification of the genomic material from lysed blood samples. The negative predictive value was 81% compared to lysed blood samples. The Kappa value for the agreement between identifying *T. brucei* s.l. using TBR-PCR in whole and lysed blood samples was calculated to be 0.1.

4.4.2.1.2 Proportion of positive discs from lysed blood samples

A total of 16 samples were found positive by application of TBR-PCR on 10 discs from lysed blood samples and another 10 discs from whole blood samples. The gold standard (defined as a positive PCR result from amplifying trypanosome DNA from whole blood and/or lysed blood applied on FTA®cards) was used to estimate the sensitivity of examining an increasing number of discs from lysed blood samples applied on FTA®cards. Table 4.7 shows the cumulative number of discs examined using TBR-PCR with their sensitivity, negative predictive value (NPV) and Kappa value compared to the gold standard.

Table 4.7: Sensitivity of cumulative number of examined discs from lysed blood samples compared to gold standard

Examined discs	One	Two	Three	Four	Five	Six	Seven	Eight	Nine	Ten
Number of positive samples	5	5	9	9	13	13	13	13	16	16
Sensitivity compared to gold standard	31.3% (8.5-54)	31.3% (8.5-54)	56.3% (31.9-80.6)	56.3% (31.9-80.6)	81.3% (62.1-100)	81.3% (62.1-100)	81.3% (62.1-100)	81.3% (62.1-100)	100% (100-100)	100% (100-100)
Negative predictive value	85.3% (77.3-93.3)	85.3% (77.3-93.3)	90.1% (83.2-97.1)	90.1% (83.2-97.1)	95.5% (90.6-100)	95.5% (90.6-100)	95.5% (90.6-100)	95.5% (90.6-100)	100% (100-100)	100% (100-100)
Kappa value	0.4	0.4	0.6	0.6	0.7	0.7	0.7	0.7	1	1

Gold standard diagnosed 16 samples to be positive using TBR-PCR reaction

The results in Table 4.7 show that with increasing the number of examined discs containing lysed blood, the sensitivity of amplifying *T. brucei* s.l. genomic material increased until reaching 100%. Examining one to two discs from lysed blood resulted in fair agreement compared to the gold standard, while increasing the number of examined discs to 3-8 resulted in moderate (0.6) and good (0.7) agreement until reaching very good agreement by examining 9-10 discs.

Looking at the number of positive discs from each sample containing whole and lysed blood, Table 4.8 shows the number of positive discs out of the 10 discs examined from each sample.

Table 4.8: Number of positive discs using TBR-PCR from the 16 positive samples (whole and lysed blood)

Sample number	Whole blood discs	Lysed blood discs
1	0/10	2/10
2	0/10	2/10
3	0/10	2/10
4	0/10	2/10
5	0/10	2/10
6	0/10	4/10
7	0/10	4/10
8	0/10	6/10
9	0/10	6/10
10	0/10	6/10
11	0/10	6/10
12	0/10	10/10
13	0/10	10/10
14	0/10	10/10
15	0/10	10/10
16	2/10	10/10
Total	2/160	92/160

The aforementioned results indicated that out of 160 discs examined from the entire whole blood samples, only two were positive for *T. brucei* s.l. DNA (1.3%). However, out of 160 discs containing lysed blood, 92 (57.5%) discs were positive for *T. brucei* s.l. The proportion of positive discs containing lysed blood was significantly higher than those contained whole blood (χ_1^2=122.01, p<0.001).

4.4.2.2 Large scale field lysis application

The results from the preliminary in field lysis application were promising regarding the improvement for identification of trypanosome DNA from FTA®card based sampling using PCR. For further evaluation of the lysis approach, another in field study was conducted on a larger scale by sampling a higher number of animals and comparing different sample preparations.

Three different sample preparations were collected from 300 animals. These preparations included, whole blood applied on FTA®cards, lysed blood on FTA®cards and in field purification of DNA from blood using extraction kits. The results from the preliminary in field lysis study indicated the improvement of identifying animals infected with *T. brucei* s.l. using TBR-PCR on lysed blood

samples by increasing the number of examined FTA discs to 9-10 discs. However, using a single PCR reaction for each disc was very expensive and time consuming. Therefore, in the large in field lysis study, ten discs from each FTA®card applied sample were examined using one PCR reaction. This was achieved by eluting the genomic material from 10 discs by heating at 90ºC using Chelex®100 resin. Moreover, another 10 discs were examined from each sample preparation using separate PCR reaction on each disc.

4.4.2.2.1 Sensitivity of cumulative number of examined discs containing whole and lysed blood

Ten discs containing whole blood samples and another 10 discs containing lysed blood samples were examined using a separate TBR-PCR reaction, the sensitivity of increasing the number of examined discs was calculated compared to the gold standard (defined as PCR positive results from amplifying trypanosome DNA from samples applied to FTA®cards and/or positive PCR results from amplifying trypanosome genomic material in DNA extract). Table 4.9 summarises the sensitivity of increased number of examined discs containing whole and lysed blood compared to the gold standard. The negative predictive value and Kappa value are also shown.

Table 4.9: Sensitivity of cumulative number of examined discs from whole and lysed blood samples compared to gold standard

Whole blood samples										
Examined discs	One	Two	Three	Four	Five	Six	Seven	Eight	Nine	Ten
Number of positive samples	6	6	6	6	6	9	18	26	34	36
Sensitivity compared to gold standard	5.9% (1.3-10.6)	5.9% (1.3-10.6)	5.9% (1.3-10.6)	5.9% (1.3-10.6)	5.9% (1.3-10.6)	8.9% (3.4-14.5)	17.8% (10.4-25.3)	25.7% (17.2-34.3)	33.7% (24.4-42.9)	35.6% (26.3-45)
Negative predictive value	67.7% (62.3-73.1)	67.7% (62.3-73.1)	67.7% (62.3-73.1)	67.7% (62.3-73.1)	67.7% (62.3-73.1)	68.4% (63.1-73.7)	70.6% (65.2-75.9)	72.6% (67.3-77.9)	74.8% (69.6-80)	75.4% (70.2-80.6)
Kappa value	0.08	0.08	0.08	0.08	0.08	0.1	0.2	0.3	0.4	0.4
Lysed blood samples										
Number of positive samples	13	13	16	18	20	22	34	41	52	74
Sensitivity compared to gold standard	12.9% (6.3-19.4)	12.9% (6.3-19.4)	15.8% (8.7-23)	17.8% (10.4-25.3)	19.8% (12-27.6)	21.8% (13.7-29.8)	33.7% (24.4-42.9)	40.6% (31-50.2)	51.5% (41.7-61.2)	73.3% (64.6-81.9)
Negative predictive value	69.3% (64-74.7)	69.3% (64-74.7)	70.1% (64.7-75.4)	70.6% (65.2-75.9)	71.1% (65.8-76.4)	71.6% (66.3-76.9)	74.8% (69.6-80)	76.8% (71.7-82)	80.2% (75.3-85.2)	88.1% (83.8-92.3)
Kappa value	0.1	0.1	0.2	0.2	0.2	0.3	0.4	0.5	0.6	0.8

Gold standard identified 101 samples to be positive using TBR-PCR reaction

A total of 101 animals were diagnosed as infected with *T. brucei* s.l. by examining different sample preparations including whole blood on FTA®cards, lysed blood on FTA®cards and DNA extract from whole blood (33.7%, 95% CI: 28.3-39.3). The results in Table 4.9 show the increase in the sensitivity

of amplifying trypanosome genomic material by increasing the number of examined discs containing whole and lysed blood. Examining one to seven discs containing whole blood resulted in poor agreement compared to the gold standard, while fair agreement was observed when examining eight to ten discs using separate PCR reactions on each disc containing whole blood.

The results in Table 4.9 show also the increase in the sensitivity of amplifying trypanosome genomic material by increasing the number of examined discs containing lysed blood samples. Examining one to five discs resulted in poor agreement compared to the gold standard, while fair agreement was observed when testing six to seven discs and moderate agreement was found by examining eight to nine discs, and good agreement was noticed by examining ten discs using separate PCR reactions on each disc.

4.4.2.2.2 Sensitivity of different sample preparations compared to the gold standard

The different sample preparations collected from the 300 animals included whole blood on FTA®cards, lysed blood on FTA®cards and DNA extract from whole blood were compared to a gold standard (defined as PCR positive results from amplifying trypanosome DNA from samples applied to FTA®cards and/or positive PCR results from amplifying trypanosome genomic material in DNA extract) to determine the sensitivity of each preparation in identifying *T. brucei* s.l. DNA using TBR-PCR. The gold standard was able to identify 101 animlas to be infected with *T. brucei* s.l. by PCR any of the used preparations.

Table 4.10: Sensitivity of the used sample preparations compared to the gold standard for identifying *T. brucei* s.l. by PCR (n=300)

Material	True positive	False positive	True negative	False negative	% NPV (95%CI)	% Sensitivity (95%CI)	Kappa value
Whole blood on FTA®cards (10 separate discs)	36	0	199	65	75.4% (70.2-80.6)	35.6% (26.3-45)	0.4
Whole blood on FTA®cards (elution)	57	0	199	44	81.9% (77.1-86.7)	56.4% (46.8-66.1)	0.6
DNA extract	69	0	199	32	86.1% (81.7-90.6)	68.3% (59.2-77.4)	0.7
Lysed blood on FTA®cards (elution)	74	0	199	27	88.1% (83.8-92.3)	73.3% (64.6-81.9)	0.8
Lysed blood on FTA®cards (10 separate discs)	74	0	199	27	88.1% (83.8-92.3)	73.3% (64.6-81.9)	0.8

Table 4.10 shows the sensitivities, negative predictive value and Kappa value of different sample preparations in amplifying *T. brucei* s.l. genomic material using TBR-PCR. The results show that the sensitivity of a single PCR reaction using 5 µl of DNA eluted from 10 discs with Chelex®100 solution

was 1.6 times higher than the amplification of the DNA from 10 separate discs of the same sample, which was statistically significant (χ_1^2=7.3, p=0.007).

Lysed blood samples were also screened for *T. brucei* s.l. genomic material using TBR-PCR on eluate from 10 discs and from another 10 discs screened separately. The sensitivity of detecting *T. brucei* s.l. DNA from lysed blood eluate was the same as screening 10 separate discs from the same sample.

Comparing the sensitivity of examining eluates either from whole, lysed blood or *in situ* DNA extract, it was found that PCR on 5 µl lysed blood eluate was 1.3 times higher than PCR on 5 µl whole blood eluate, however this difference was statistically insignificant (χ_1^2=1.7, p=0.2). Moreover, the sensitivity of PCR on 5 µl lysed blood eluate from 10 discs using Chelex®100 was 1.1 higher than PCR on 1 µl from whole blood DNA extract. Therefore, these single PCR reactions on eluate released from material stored on FTA®cards are comparable to DNA prepared directly under field conditions at the point of collection.

The results in the table show also the Kappa value for the degree of agreement between the amplification of *T. brucei* s.l. genomic material in different preparations compared to the gold standard. There was fair agreement between the detection of the DNA in whole blood samples using separate 10 discs for the PCR and the gold standard. Moderate agreement was noticed when amplifying the parasite genomic material from eluates of whole blood discs. However, very good agreement was found between the use of lysed blood eluate, 10 separate discs contained lysed blood and the gold standard. Good agreement was also observed in the amplification of the trypanosome genomic material using DNA extract compared to the gold standard.

4.5 Discussion

4.5.1 Experimental lysis of blood samples

Previous work had demonstrated that when infected blood is applied to the surface of the FTA®card, parasitic material is localised at the point of contact (Cox, 2007). Therefore, at low parasitaemia much of the FTA®card surface must be screened in order to confirm the infective status of the animal.

The aim of this chapter was to demonstrate that by lysing blood, it is possible to create a more homogenous solution of DNA consisting of the genetic material from both the host as well as any infectious agent prior to application on FTA®cards.

Application of the lysis agents (H_2O, PBS and 1% SDS) to procyclic cultures improved the detection of the trypanosome DNA. This improvement was attributed to the rupture of the procyclic cells due to osmotic pressure resulting from addition of the lysis agents leading to release of the genomic material into the solution. This release of the DNA resulted in a more homogenous distribution of the

trypanosome DNA across the surface of the FTA®card, increasing the chances of detecting the trypanosome genomic material by PCR.

Three experimental lysis trials were performed using procyclic and bloodforms of *T. brucei* s.l. in order to improve the downstream sensitivity of PCR screening of samples stored on FTA®cards. The first trial was conducted using procyclic forms in Cunningham's media with concentrations ranging from $2.5x10^5$ to 2.5 parasites/ml. Samples prior to lysis were examined using a PCR that specifically targets a multi-copy region within the genome of *T. brucei* s.l.; the results indicated that at high concentrations ($2.5x10^5$- $2.5x10^2$ parasites/ml) there were sufficient trypanosomes on the card matrix to be amplified with the PCR reaction. However, at lower concentrations (25-2.5 parasites/ml) the chance of isolating a parasite in each of the examined disc was lower (3% and 0.3%, respectively), therefore, it was unsurprising that none of the examined discs from these low concentrations contained the trypanosomes.

The choice of a PCR that targets a high copy number sequence also improved the detection of the DNA from as low as one parasite per ml. For instance, at the concentration containing 25 parasites/ml; 50 µl from this concentration would contain one parasite. Lysing the cell using 50 µl of lysis agent resulted in the release of the genomic material from the parasitic cell into the solution. The copy number of the target in one trypanosome cell is 10,000 copies; therefore, this copy number of the target exists in 100 µl lysed sample applied on FTA®card. One disc from FTA®card is equivalent to 1.34 µl, so examining one disc from such solution after application on FTA®cards resulted in the amplification of the parasite DNA because it was assumed that one disc would contain 134 copies of the target. This assumption clarifies the detection of the trypanosome DNA at low concentrations after lysing the cells.

Lysis was also successful in the second trial, however, the number of discs reacting positive by PCR was lower compared to the first trial. This could be attributed to masking of the parasite DNA by the presence of host DNA. The DNA content in the 50 µl volume of the host blood sample was reported to be 1.6 µg (Qiagen extraction kits, 2007), so this amount was added to match the natural conditions where both the parasitic and host DNA co-exist in the sample. The lysis experiment was repeated using bloodforms from a mouse blood. This trial further indicated that lysing the cells prior to their application onto FTA®cards, especially at the low parasite concentration improved the trypanosome diagnosis by PCR. However, the number of the discs reacting positive by PCR was again less compared to the first trial which again suggests the masking of the parasite genomic material with the host DNA.

These results are in agreement with those obtained by Moser *et al*. (1989a) who stated that the presence of up to 1 µg of mouse or human DNA with the trypanosome DNA in the PCR reaction did not affect the sensitivity of parasite DNA detection. In the current study, the concentration of the host DNA was 60% higher than the acceptable concentration reported by Moser *et al*. (1989a).

The presence of human DNA is an important factor that interferes with the reaction yield of the PCR; this was described by Contamin *et al*. (1995) who noticed a decrease of the PCR sensitivity in identifying malaria parasites in human blood by 10 fold due to the presence of human DNA. Cogswell *et al*. (1996) reported that a diagnostic PCR can be inhibited if the amount of host DNA exceeds a certain threshold. In their system, this value was calculated to be > 0.5 µg of host DNA. The possible explanation for this masking effect is that the host DNA is able to competitively inhibit primer-template hybridization when the host DNA is present at high concentration due to the short sequence of primers which allows identities to be found with some of the host DNA sequences (Cogswell *et al.*, 1996).

Regarding the lysing agents used, water was chosen for the in field application due to the availability, low cost and unnecessary pre-use preparations.

4.5.2 In field application of lysis

The experimental lysis trials clarified the possibility of increasing the chance of trypanosome DNA identification using PCR by pre-treating the blood releasing the genomic material in a more homogenous solution prior to application to FTA®cards. The lysis trial was then applied under field conditions to help evaluating this approach as a tool for field application.

4.5.2.1 Preliminary field lysis trial

The preliminary field lysis trial was a pilot study to obtain initial information about the logistics of lysis in the field; therefore, opportunistic samples were collected from 80 cattle at Lwala parish, Olemai village in Kabermaido district, Eastern Uganda, in July, 2006.

Whole and lysed blood samples were collected from 80 cattle and 10 separate discs from each preparation were examined by PCR directed against *T. brucei* s.l., this is the same PCR protocol that was used in the experimental trials. The overall PCR results showed that examining lysed blood samples indicated 20% of the animals were infected with *T. brucei* s.l., while examining whole blood samples identified only 1.3% to be infected. To discuss these findings, let us assume that the 100 µl whole blood sample collected from the animal contained two parasites; application of this amount on FTA®card matrix would entrap these parasite cells in a certain location on the matrix, for the animal to be diagnosed positive, the examined disc should contain a parasite cell. However, assuming that 50 µl of the whole blood collected from the animal contained one parasite, after adding the lysis agent the cell ruptured and the genomic material released into the solution. After application of the lysis solution to FTA®cards, examining just one disc should contain sufficient parasitic material to produce a positive PCR.

Increasing the number of discs examined improved the detection further due to increasing the chance of selecting a region of the card where parasitic material was stored. This was clearly noticed from comparing the sensitivity of cumulative number of examined discs to the gold standard (defined as a positive PCR result from amplifying trypanosome DNA from whole blood and/or lysed blood applied on FTA®cards).

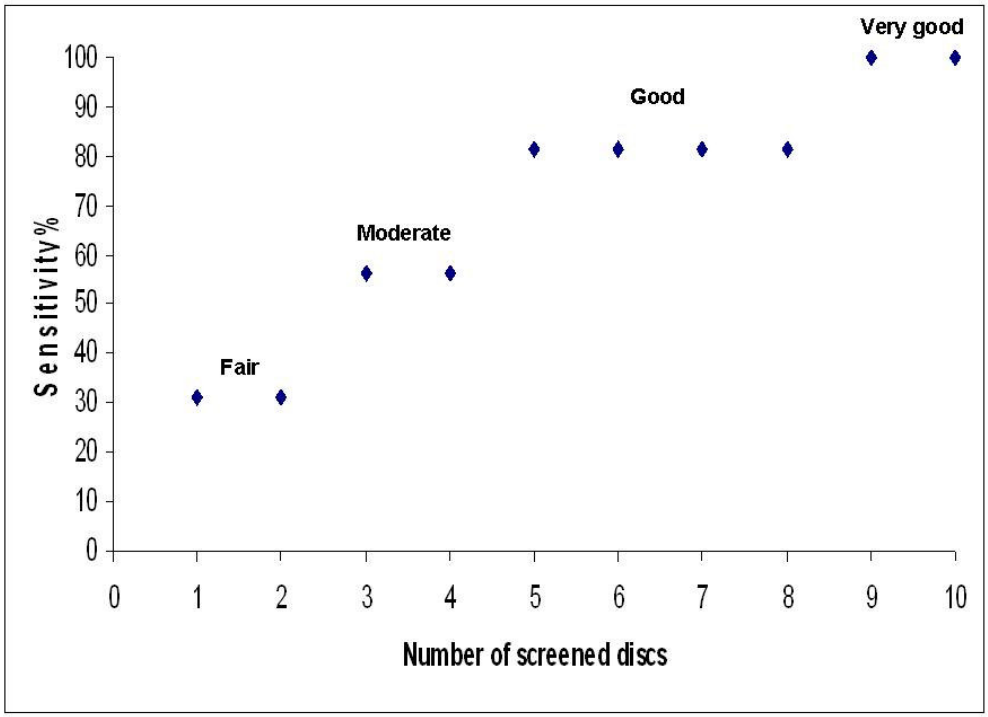


Figure 4.5: Cumulative number of examined discs containing lysed blood (the level of Kappa agreement is shown between brackets according to Altman (1991))

Figure 4.5 shows that increasing the number of the examined discs containing lysed blood samples to 10 discs resulted in 100% sensitivity compared with the gold standard. The steep increase in the sensitivity would suggest that although the distribution is improved by pre-lysis, it should not be assumed to be a homogenous distribution; by increasing the number of screened discs it is possible to improve the detection of trypanosome DNA.

Looking closely at the results of the examined discs from the 16 positive animals, examining 10 discs from whole and lysed blood samples resulted in a significantly higher proportion of lysed blood discs identified positive for the trypanosome target compared to whole blood examined discs. The results from the pilot study confirmed that the lysis step did indeed improve the sensitivity of trypanosome DNA detection from infected cattle blood. However, this level of sensitivity required the examination

of nine to ten discs using a separate PCR reaction on each disc which is time consuming and expensive. Therefore, a larger field lysis study was conducted for further evaluation of the approach.

4.5.2.2 Large scale field lysis trial

For further evaluation of the lysis approach, a large scale field study was conducted using a larger sample number (n=300) and three types of sample preparations. The sample preparations used in this study were whole blood on FTA®cards, lysed blood on FTA®cards and *in situ* DNA extraction.

Starting with examining 10 separate discs from FTA®card applied samples, the sensitivity of detecting positive discs containing whole blood was constant at 5.9% using one to five discs, while the sensitivity was increased using more discs until reaching 35.6% after examining 10 discs using PCR (Figure 4.6). This was due to increasing the chance of obtaining the parasite genomic material in the examined disc. However, using discs containing lysed blood samples, the sensitivity of detecting positive discs using PCR increased from 12.9% using one to two discs to 73.3% using 10 discs. Comparing the sensitivity of examining one disc containing whole blood and lysed blood (5.9% and 12.9%, respectively), it was noticed that the lysis step doubled the sensitivity of the screening protocol; also this was the case in comparing the sensitivity of examining 10 discs from the two preparations using separate PCR reactions (35.6% and 73.3%, respectively).

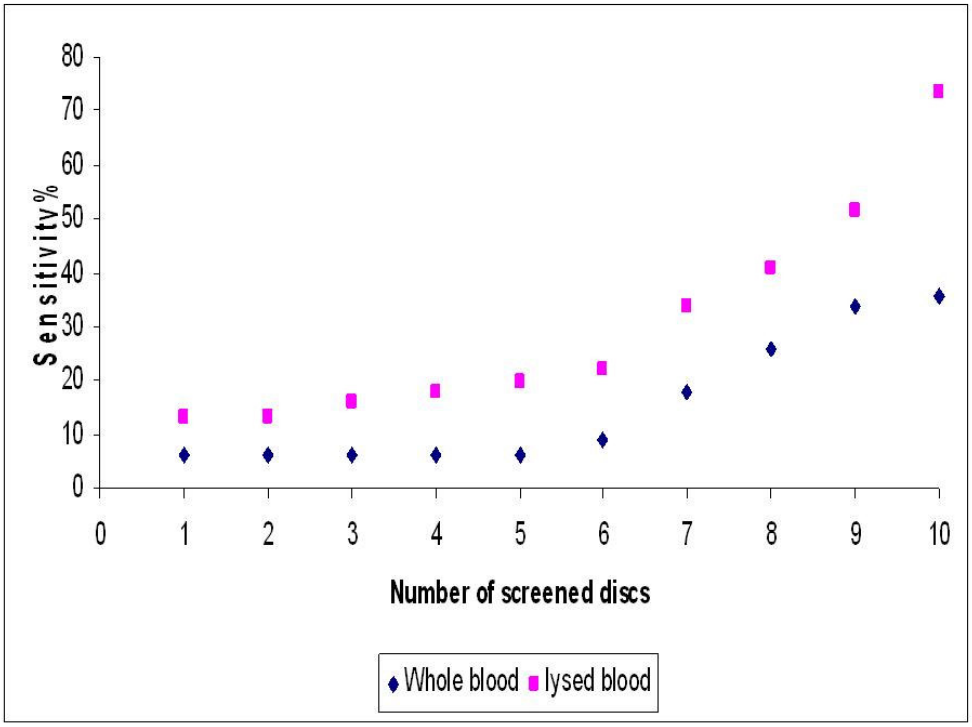


Figure 4.6: Cumulative number of examined discs containing whole and lysed blood

The improvement of detecting trypanosome target sequence was clear using the lysed blood samples; however, it required the screening of at least 10 separate discs to achieve a sufficient sensitivity. The prevalence obtained from a single disc may be completely representative of the population if infection intensities are generally very high, or may completely underestimate the population prevalence if the infection intensities are generally very low in the population (Cox, 2007). Therefore, in order to decrease the probability of false negative results from using a single disc, examining more discs would give more accurate estimation of the disease prevalence.

Although the higher sensitivity of examining more discs, the use of 10 discs from whole blood or lysed blood samples with a separate PCR reaction on each disc was considered time consuming and expensive. Moreover, the use of 10 discs for detecting each trypanosome species would quickly use all the archived material on the card matrix. Therefore, to improve the detection of trypanosomes using only 10 discs with one PCR reaction for each species, another 10 discs from whole and lysed blood preparations were examined using one PCR reaction. This was achieved by eluting DNA from FTA®cards containing whole blood and lysed blood using 5% Chelex®100 resin aqueous suspension (Becker *et al.*, 2004).

Genomic material in the 10 discs of whole and lysed blood was eluted in 60 µl Chelex®100 suspension solution, 5 µl eluate was subjected to PCR amplification using TBR-PCR. This volume of the eluate would be sufficient for about 12 PCR reactions. The results showed that the sensitivity of identifying trypanosome DNA from whole blood and lysed blood eluate was 56.4% and 73.3%, respectively, compared to the gold standard (Figure 4.6).

The sensitivity of lysed blood eluate in detecting trypanosome DNA was insignificantly higher than the eluate obtained from the whole blood samples (χ_1^2=2.8, p=0.1). Although the difference was insignificant, the increased proportion of animals diagnosed to be infected with *T. brucei* s.l. using lysed blood preparations supported the conclusion obtained from the initial field lysis analysis and the experimental lysis trials that lysing trypanosome cells increased the probability of the target detection. Therefore, the improved distribution of the genomic material on the FTA®card matrix gives a greater possibility of detecting trypanosomes than in whole blood applied directly on FTA®cards.

In situ DNA extraction from 50 µl blood sample was compared to the gold standard for the sensitivity of detecting the parasite DNA when a single µl was examined by PCR. Assuming that the collected volume of blood contain a single trypanosome cell, after sample lysis, the resulting solution becomes "contaminated" by the parasite DNA with 10,000 copies per genome of the target. This contamination is such that you would expect every 1 µl of DNA extract examined thereafter by PCR to be positive, because it would, theoretically, contain 200 copies of the target sequence. However, the sensitivity of detecting the parasite target sequence in the DNA extract was insignificantly lower than that obtained using eluate from lysed blood discs (χ_1^2=0.2, p=0.6).

DNA extraction from different samples including blood, CSF, tissues and cultures is an important step for conducting PCR for the diagnosis and research purposes. Extraction of DNA using different kits has been proven to recover high molecular weight DNA but this process is time consuming and expensive. Moreover, these methods require several steps and may include the transfer of DNA extracts to additional containers and columns increasing the chance for cross-transfer of samples or the introduction of contaminants (Walsh *et al.*, 1991). ChargeSwitch® gDNA kit used in this study to extract DNA from blood did not require the use of any electrical equipment because it depends on the use of magnetic beads, however, it was time consuming and expensive. For extracting DNA from one sample using ChargeSwitch® gDNA kit, the cost was £4.20 compared to £2.70 and £3.20 using whole blood and lysed blood on FTA®cards, respectively.

Research has focused on the development of alternative extraction protocols that overcome the drawbacks of the conventional methods, but at the same time producing efficient DNA yield in both the quantity and quality to be easily amplified using PCR (Polski *et al.*, 1998). DNA elution from blood applied on FTA®cards or filter papers using 5% aqueous Chelex®100 solutions appear to be more convenient and cheaper than DNA extraction using extraction kits (Becker *et al.*, 2004). This is because the use of Chelex®100 has decreased the number of steps in sample preparation for DNA extraction, thus decreasing the possibility of cross transfer and contamination of samples (Walsh *et al.*, 1991).

In conclusion, successful molecular diagnosis requires the availability of genomic material of an appropriate quality and concentration to be present within the sample under examination. The insignificant difference between using DNA eluted from whole blood, lysed blood discs and DNA extracted using kits suggests to use any of the aforementioned preparations for obtaining trypanosome genomic materials. The choice will be based on economic and time factors, in field DNA extraction using kits will be excluded due to the expensive kits and time consumed for extracting DNA. FTA®card technology is an important archiving matrix for DNA, pre-application lysis of infected blood products can help create a more homogenous distribution. It is possible to elute DNA from FTA®cards; this approach overcomes the uneven distribution of materials across the matrix. The pre-lysis step further improves the detection of trypanosomes in this eluted preparation to an acceptable level of accuracy.

5 Chapter five:

Evaluation of different PCR reactions in use for trypanosome diagnosis

5.1 Introduction

Diagnosis of human and animal African trypanosomiasis has been the subject of extensive research since the discovery of the disease by Bruce in 1903. The most important reason for trypanosome diagnosis is for the appropriate application of effective control measures and management of the disease (Eisler *et al.*, 2004; Molyneux, 1975). The routine diagnosis of trypanosomiasis using parasitological approaches has been reported to have poor sensitivity under field conditions due to low peripheral parasitemia in infected animals (Masake *et al.*, 2002; Picozzi *et al.*, 2002).

The drawbacks of microscopy led scientists to turn their attention towards the detection of antibodies against the infectious agent as an alternative means of diagnosing infection. Serological tests such as complement fixation test (CFT), indirect fluorescent antibody test (IFAT), card agglutination test (CATT) and enzyme linked immunosorbant assay (ELISA) have been used for the diagnosis of trypanosomes (Luckins, 1977). However, these methods are unable to differentiate between existing and previous exposure to infection (Bengaly *et al.*, 1995). This has led the attention to be directed towards the identification of the infectious agent DNA for diagnosis using the polymerase chain reaction (PCR).

In recent years, PCR has been developed and widely used for the detection of trypanosomes (Gonzales *et al.*, 2003; Moser *et al.*, 1989a). PCR has proven to be highly sensitive and specific for trypanosome detection (Becker *et al.*, 2004). The use of PCR in detecting trypanosome DNA seems to be the most reliable and accurate technique to assess the efficacy of trypanosomiasis treatment by the specific identification of natural animal infections with most trypanosome species and sub-species (Njiokou *et al.*, 2004; Truc *et al.*, 1999).

Different types of PCR are in use for the diagnosis of trypanosomes, including conventional, multiplex and nested reactions. Conventional PCR reactions mainly depend on the use of primer sets targeting a specific DNA sequence. In multiplex reactions, more than one primer set can be used which allows more than one DNA sequence to be targeted. Finally, nested PCR depends on the run of two separate reactions where the amplification product from the first reaction is used as a template for the second reaction.

The diagnosis of the most important pathogenic trypanosome species (*T. brucei* s.l., *T. congolense* and *T. vivax*) has been achieved using PCRs designed for species-specific targets. The most favourable target for identifying *T. brucei* s.l. is the satellite repetitive 177 bp sequence described by Sloof *et al.* (1983a,b) due to the high copy number in the parasite genome (10,000). The sensitivity of the primers designed to target the sequence is detailed in Chapter II (section 2.2.5.2.1).

The Savannah type of *T. congolense* is highly pathogenic and is the most widespread, in terms of both geographical and host range (Bengaly *et al.*, 1995; Gibson *et al.*, 1988; Young and Godfrey, 1983).

The specific primers designed for this species targeting a satellite sequence of 316 bp were designed by Moser *et al*. (1989a) and the sensitivity of the primers are detailed in Chapter II (section 2.2.5.2.1).

Universal primers targeting a fragment of the gene encoding *T. vivax* specific antigen were described by Masake *et al*. (1994). The antigen is recognised by a monoclonal antibody (Tv27) in an Ag-ELISA, the cloned gene was found to be tandemly repeated with a monomeric unit length of 900 bp in the genome of all *T. vivax* isolates from diverse geographic locations in Africa and South America (Chapter II, section 2.2.5.2.1).

A high copy number combined with inter-species length variation makes the internal transcribed spacers (ITS), located within the ribosomal RNA genes, a useful marker for species differentiation in trypanosomes and other species (Cox *et al.*, 2005; Desquesnes *et al.*, 2001; McLaughlin *et al.*, 1996; Njiru *et al.*, 2004). The development of different PCR reactions based on the amplification of the ITS region is fully discussed in Chapter II (section 2.2.5.2.2). Cox *et al*. (2005) developed a simple nested PCR method which detects the inter-species length variation of the ITS region of ribosomal genes, thereby producing a unique size of PCR product for each species of trypanosomes.

5.2 Objectives

The aim of the work presented in this chapter is to evaluate the different PCR reactions currently in use for the diagnosis of the most important pathogenic trypanosomes. The PCR reactions evaluated in this study include species-specific PCR reactions that amplify *T. brucei* s.l., *T. congolense* Savannah and *T. vivax,* compared to the pan-trypanosome nested reaction that amplifies the ITS locus in the rRNA gene (Cox *et al.*, 2005).

5.3 Material and methods

5.3.1 Collected samples

The blood samples used in the current study were collected in Uganda and Zambia from cattle, wildlife, sheep and dogs. Table 5.1 summarises the origin of the samples used. The blood samples spotted directly onto FTA®cards were analysed for the presence of trypanosome infection with a number of different PCR reactions.

Table 5.1: Description of the samples used in the current study

Area of collection	Host	Number of samples	Date of collection	Comments
Uganda	Cattle	600	October, 2006	Samples collected during baseline sampling of Stamp Out Sleeping sickness campaign
Zambia	Wildlife	276	July, 2005	Kindly provided by Neil Anderson
	Sheep	58	October, 2005	Kindly provided by Joseph Matemba
	Dogs	35	October, 2005	
Total		969		

5.3.2 Samples processing

Samples applied onto FTA®cards were processed by eluting DNA from 10 discs (0.2 mm) using Chelex®100 as previously described in Chapter IV (section, 4.3.2.2.3). The same eluate was used for all the PCR reactions on each sample to make these reactions comparable.

5.3.3 PCR

The primer sequences, reaction conditions and the size of the amplified products of ITS-PCR and TBR-PCR are detailed in chapters III and IV, respectively. The primer sequence, reaction conditions and size of the amplified products using PLC-PCR, TCS-PCR, TCK-PCR, TCF-PCR, universal TV-PCR and West African TV-PCR are listed in Table 5.2.

Table 5.2: Primer sequences, reaction conditions and size of amplified products using species-specific PCR

PCR	Diagnosed species	Product size (bp)	Primer sequence	Cycles number	Reaction conditions	Reference
PLC-PCR	*T. brucei* s.l.	324 bp	657: 5'- CGC TTT GTT GAG GAG CTG CAA GCA-3' 658: 5'- TGC CAC CGC AAA GTC GTT ATT TCG-3'	40	94ºC for 30 sec, 63ºC for 90 sec, 72ºC for 70 sec.	(Picozzi *et al.*, 2008)
TCS-PCR	*T. congolense* Savannah	316 bp	TCS1: 5'-CGA GAA CGG GCA CTT TGC GA-3' TCS2: 5'-GGA CAA ACA AAT CCC GGGCA CA-3'	35	94ºC for 1 min, 55ºC for 2 min, 72ºC for 2 min.	(Moser *et al.*, 1989a)
TCK	*T. congolense* Kilifi	294 bp	TCK1: 5'- GTG CCC AAA TTT GAA GTG AT-3' TCK2: 5'- ACT CAA AAT CGT GCA CCT CG-3'	35	94ºC for 1 min, 55ºC for 2 min, 72ºC for 2 min.	(Masiga *et al.*, 1992)
TCF	*T. congolense* Forest	350 bp	TCF: 5'- GGA CAC GCC AGA AGG TAC TT-3' TCF2: 5'- GTT CTC GCA CCA AAT CCA AC-3'	35	94ºC for 1 min, 55ºC for 2 min, 72ºC for 2 min.	(Masiga *et al.*, 1992)
Universal TV-PCR	*T. vivax*	400 bp	ILO1264: 5'-CAG CTC GGC GAA GGC CAC TTC GCT GGG GTG-3' ILO1265: 5'-TCG CTA CCA CAG TCG CAA TCG TCG TCT CAA GG-3'	35	94ºC for 1 min, 55ºC for 2 min, 72ºC for 2 min.	(Masake *et al.*, 1997)
West African TV-PCR	*T. vivax*	175 bp	TVW1: 5'-GTG CTC CAT GTG CCA CGT TG-3' TVW2: 5'-CAT ATG GTC TGG GAG CGG GT-3'	35	94ºC for 45 sec, 60ºC for 1 min, 72ºC for 30 sec.	(Masake *et al.*, 1994)

Generally, the reaction volume used was 25 µl containing NH_4 Buffer (Bioline, London, UK) that is formed of 16.0 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25ºC) and 0.01% Tween 20. Moreover, 1 µM of each primer, 1.5 mM $MgCl_2$, 200 µM of each of the four-deoxynucleoside triphosphates (dNTP) and one Unit of BIOTAQ RED DNA Polymersase (Bioline) were added.

PLC-PCR reaction mix routinely contained 1.5 U Hot Star Taq, 1.25 µl of Rediload dye (Invitrogen) with a final concentration of 3 mM $MgCl_2$ and 0.2 µM of each primer.

After preparing the master mix, 5 µl of the eluate was added to 20 µl of the PCR master mix. The reaction conditions for the individual PCR are summarised in Table 5.2, an initial denaturation step at 94ºC for 3 minutes was used together with a final elongation step at 72ºC for five minutes except for PLC-PCR this step was for 10 minutes. Also, for PLC-PCR the initial denaturation was at 95ºC for 15 minutes to activate Hot Start *Taq* polymerase. The reactions took place in a DNA Engine DYADTM Peltier Thermal Cycler. Visualization of the amplified bands was detailed in Chapter three (section

3.4.7.3) with an example of ITS-PCR and TBR-PCR gel results. Figure 5.1 shows an example of the universal TV-PCR, TCS-PCR and PLC-PCR gel results.

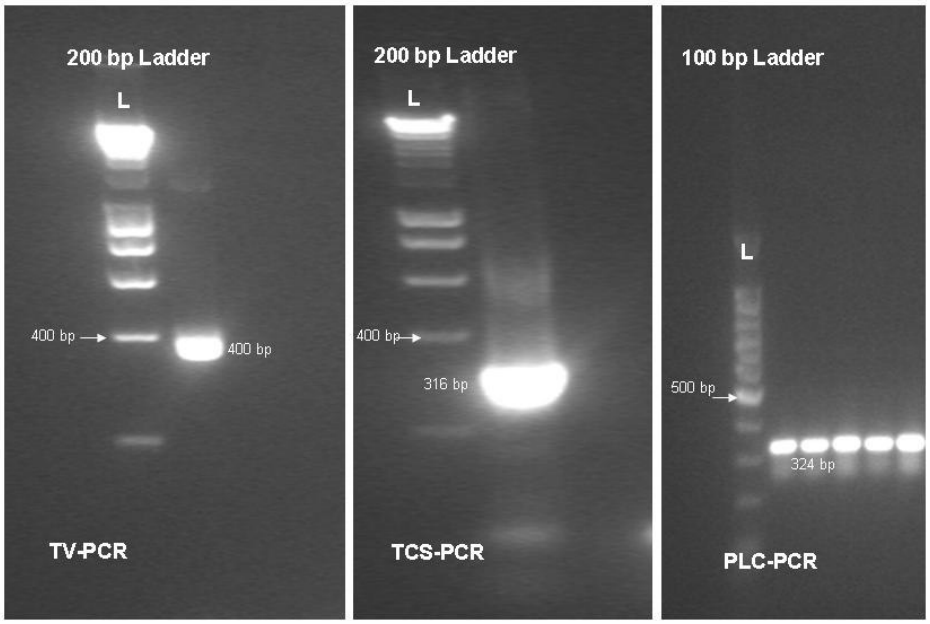


Figure 5.1: Example of the universal TV-PCR, TCS-PCR and PLC-PCR gel results (size of the amplified product in each reaction is shown in the image, L: Ladder)

5.3.4 Cloning protocol

The amplified products from ITS-PCR reaction used in this study were cloned and sequenced to further evaluate the specificity of the PCR reaction.

5.3.4.1 Gel extraction

Gel extraction of the desired fragment was carried out with MinElute gel extraction kits (Qiagen), using spin-columns designed to give high end concentrations of purified DNA fragments. The main principle of these kits is the adsorption of DNA to the silica-gel membrane in the presence of a high chaotropic salt concentration, contaminants pass through the column with washing of impurities and elution of the pure DNA with Tris buffer. The silica-gel membrane is designed to adsorb up to 5 µg DNA allowing the purification of concentrated DNA fragments in high yields, in as little as 9 µl eluate. The adsorption of DNA to silica depends on pH with 80% recovery of DNA when the loading

mixture pH is ≤7.5. QG buffer is a binding and solubilisation buffer which solubilises the agarose gel slice and provides the appropriate conditions for binding of DNA to the silica membrane. The pH indicator in QG buffer appears yellow at pH ≤7.5, but in cases of pH> 7.5 which occurs due to running the gel in repeatedly used electrophoresis buffer or incorrectly prepared buffer, the binding mixture turns orange or violet leading to inefficient binding of the DNA to the silica-gel membrane. In this case, the pH can be corrected by adding a small volume of 3 M sodium acetate (pH 5). The indicator dye does not interfere with DNA binding and is removed during the cleanup procedure.

During the DNA adsorption step, unwanted primers and impurities (salts, enzymes, unincorporated nucleotides, agarose, dyes, ethidium bromide, DMSO, oils and detergents) do not bind to the silica membrane but flow through the column. Salts are washed away with the ethanol-containing buffer PE which is removed by an additional centrifugation step to avoid its interference with subsequent enzymatic reactions.

Elution efficiency is strongly dependent on the salt concentration and pH of the elution buffer, contrary to adsorption; elution is most efficient under basic conditions (pH 7-8.5) and low salt concentrations. Elution with TE is possible but not recommended because EDTA may inhibit subsequent enzymatic reactions. Also, elution in water is possible, but DNA should be stored at -20ºC since DNA may degrade in the absence of a buffering agent.

The desired DNA fragment was excised from the agarose gel with a clean, sharp scalpel, and weighed in a colourless tube (the maximum amount of gel slice per spin column is 400 mg). Buffer QG was added as 3 volumes to one volume of the gel (<2%), but for gels >2%, 6 volumes of the buffer were added.

The tubes containing the gel slice and QG buffer were incubated in a water bath at 50ºC for 10 minutes or until the gel slices were completely dissolved. Isopropanol was added as one gel volume and the tubes were mixed by inversion several times. To bind the DNA, the samples were applied to the MinElute spin column and centrifuged for one minute at 13,000 rpm. The flow through was discarded and 500 µl of buffer QG was added to the spin column and centrifuged for one minute at 13,000 rpm. To wash away the impurities, 750 µl of buffer PE was added to the column and left to stand at room temperature for five minutes before centrifugation at 13,000 rpm for one minute. After discarding the flow through, the tubes were additionally centrifuged for one minute at 13,000 rpm to remove the residual ethanol. For eluting DNA, the spin column was replaced into a clean 1.5 ml micro-centrifuge tube and 10 µl of buffer EB was added to the centre of the membrane and the tubes were left to stand at room temperature for one minute before centrifugation at 13,000 rpm for one minute.

5.3.4.2 Cloning

Qiagen PCR Cloning kits are considered a highly efficient technology for cloning of PCR products using *Taq* and other non-proofreading DNA polymerases. The supplied vector in the kits, pDrive Cloning vector (Figure 5.2), is provided in a linear form with a Uracil overhang (U) at each end to hybridise with high specificity to PCR products generated using *Taq* and other non-proofreading polymerases with a single Adenosine overhang (A) at each end. For efficient addition of an A overhang during the PCR amplification, a final extension step at 72ºC for 10 minutes is strongly recommended. The Ligation master mix provides optimal buffer conditions for the hybridisation of the pDrive vector and the PCR products.

The pDrive cloning vector is supplied in a linear form, and allows ampicillin and kanamycin selection as well as blue/white colony screening. The vector contains several unique restriction endonuclease recognition sites around the cloning site allowing easy restriction analysis of recombinant plasmids.

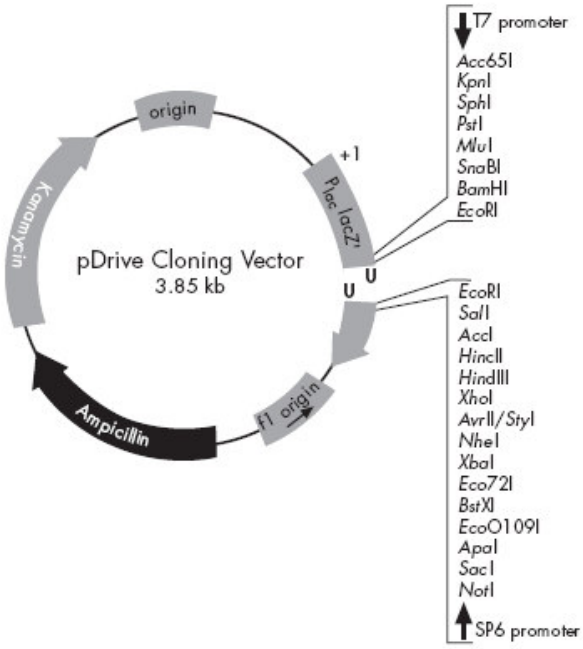


Figure 5.2: Plasmid map of pDrive cloning vector (Qiagen)

5.3.4.3 Ligation

After thawing 2x Ligation master mix and pDriver cloning vector, DNA was placed on ice and the ligation reaction mixture was prepared as follows in the same order (Ligation master mix was added last):

PCR product	4 µl
pDrive cloning vector	1 µl
Ligation master mix	5 µl
Total	10 µl

The ligation reaction mixture was briefly mixed by pipetting up and down few minutes then the mix was incubated at 4ºC for two hours.

5.3.4.4 Preparation of Agar plates

Luria Bertani (LB) Broth was prepared by dissolving 10 g tryptone, 5 g yeast extract, 10 g sodium chloride and 15 g agar in 950 ml of de-ionised water. The pH of the solution was adjusted to seven and water was added to make up the volume to 1000 ml. The solution was then autoclaved for 20 minutes at 15 lbs pressure. The autoclaved solution was cooled to 55ºC and one ml of 50 mg/ml ampicillin suspension was added and poured into sterile Petri dish. After the plates had set, 20 µl of X-Gal (50 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyransoide) and 100 µl IPTG (100 mM isopropyl β-D-thiogalactopyranoside) were added to the surface of each plate and evenly distributed using a spreader, the plates were then left to absorb these solutions at 37 ºC for 30 minutes. Cells transformed by pDrive cloning vector which do not contain a PCR product will form blue colonies when grown in the presence of X-Gal/IPTG, while, cells transformed by pDrive cloning vector which contain PCR product will form white colonies.

The plates were then inverted and stored at 4ºC till further use. It is recommended to use freshly poured plates (< one week old) for screening plates because long term storage of plates can result in decreased antibiotic activity.

5.3.4.5 Transformation

Qiagen EZ competent cells were thawed on ice, while, SOC medium [tryptone (pancreatic digest of casein) 2% (w/v), yeast extract 0.5% (w/v), NaCl 8.6 mM, KCl 2.5 mM, $MgSO_4$ 20 mM and glucose 20 mM] was thawed and allowed to warm to room temperature before being used. 2 µl of the Ligation reaction mixture was added to each tube of competent cells and mixed by gently flicking the tubes. The cells were incubated on ice for five minutes, then, the tubes were heated in a water bath at 42ºC

for 30 seconds and again incubated on ice for two minutes. SOC medium (250 µl) was then added to each tube and half of each mixture was plated on an agar plate. The plates were incubated at room temperature until the transformation mixture was completely absorbed, then they were inverted and incubated at 37ºC overnight. The next day, the plates were further incubated at 4ºC for three hours to facilitate blue/white screening.

After blue/white screening, six white colonies from each agar plate were chosen and suspended in 100 µl of LB broth containing ampicillin (100 mg/ml). The tubes were incubated at 37ºC for three hours; the presence of the DNA insert was then confirmed by PCR amplification. The bacterial colonies positive with PCR amplification were re-suspended in 3 ml of LB broth containing ampicillin suspension and incubated at 37ºC overnight.

5.3.4.6 Preparation and storage of bacterial culture stock

After incubating the bacterial culture in LB broth, some part of the bacterial culture was mixed with Glycerol (1:2) and was stored at -80ºC for future use. The plasmid DNA was extracted from the rest of the culture by using the Qiagen Miniprep kit.

5.3.4.7 Plasmid DNA purification

The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica membrane in the presence of high salt buffer and elution in low salt buffer. Bacterial cells suspended in LB broth (3 ml) were pelleted by centrifugation at 3000 rpm for 10 minutes followed by re-suspension of the bacterial pellet in 250 µl buffer P1 and transferred to 1.5 ml micro-centrifuge tube. 250 µl of buffer P2 was also added to the tube and mixed thoroughly by inverting the tube several times immediately, 350 µl of buffer N3 was added and mixed thoroughly by inverting the tube several times to avoid localised precipitation until the solution became cloudy. The solution was then centrifuged at 13,000 rpm for 10 minutes and the supernatant was pipetted to the QIAprep spin column and centrifuged for 60 seconds at 13,000 rpm. The flow-through was discarded and the spin column was washed by adding 500 µl of buffer PB followed by centrifugation at 13,000 rpm for 60 seconds and the flow-through was discarded. The spin column was washed using 750 µl buffer PE by centrifugation at 13,000 rpm for 60 seconds. The flow-through was discarded before centrifugation for an additional minute to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml micro-centrifuge tube. DNA was eluted by adding 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) at the centre of the spin column; this was left to stand for one minute then centrifuged at 13,000 rpm for another minute. Eluted plasmid DNA was stored at -20ºC until it was sent for sequencing.

5.3.5 Sequence analysis

Clones were sequenced by GATC biotechnology, Germany. Sequences were analysed using DNASTAR software and the BLAST search on the National Centre of Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?).

5.3.6 Statistical analysis

Sensitivity, negative predictive value and Kappa value were calculated as previously described in Chapter III (section, 3.4.8).

5.4 Results

The aim of the current study was to evaluate different PCR reactions for the detection of trypanosome DNA in blood samples applied on FTA®cards. Eluate from 10 discs was used to run all PCR reactions for each sample, accordingly the results are comparable. Species-specific PCR reactions used in this study were TBR-PCR, TCS-PCR, TCF-PCR, TCK-PCR and TV-PCR reactions for the diagnosis of *T. brucei* s.l., *T. congolense* Savannah, *T. congolense* Forest, *T. congolense* Kilifi and *T. vivax*, respectively.

5.4.1 Comparing ITS-PCR with species-specific PCR reactions

A total of 969 blood samples collected from different animal species on FTA®cards were examined using ITS-PCR and species-specific PCR, the parasitic events identified are summarised in Table 5.3.

Table 5.3: Number of animals infected with trypanosomes diagnosed by different PCR reactions

	***T. brucei* s.l.**	***T. congolense****	***T. vivax***	Total
ITS-PCR	2	21	24	47
Species-specific PCR	77	79	41	197

***ITS-PCR results with amplicon size more than 1400 bp were classified as *T. congolense* due to the difficulty in classifying the sizes of the three sub species**

The results in Table 5.3 show the number of infected animals with different trypanosome species using ITS and species-specific PCR. Overall, ITS-PCR detected 47 parasitic events, while species-specific PCR reactions identified 197 parasitic events, the difference was statistically significant (χ_1^2=105.5, p<0.001).

T. brucei s.l. species

Looking at the sensitivity of the different PCR reactions in the diagnosis of each trypanosome species, specific PCR for *T. brucei* s.l. identified 77 parasitic events. ITS-PCR confirmed only two animals to be infected with *T. brucei* s.l., the sensitivity of ITS-PCR and species-specific PCR was compared to a

gold standard which in this case was defined as a positive result of ITS-PCR and/or TBR-PCR in amplifying *T. brucei* s.l. DNA. The sensitivity of TBR-PCR was found to be 100% in amplifying *T. brucei* s.l. DNA compared to the gold standard with a very good agreement (Kappa value=1). However, ITS-PCR sensitivity was only 2.6% (95% CI: 0-6.2) with NPV of 92.2% (95% CI: 90.6-93.9) and poor agreement compared to the gold standard (Kappa value=0.04). Overall, TBR-PCR significantly identified more *T. brucei* s.l. infections than ITS-PCR (χ_1^2=74.2, p<0.001).

To investigate if this difference in the sensitivity was attributed to the difference in the copy number of the target, another PCR reaction specific for *T. brucei* s.l. targeting a single copy gene (PLC-PCR) was used using the same eluted DNA prepared. PLC-PCR identified 49 animals to be infected with *T. brucei* s.l. of which only two were confirmed with ITS-PCR. The results of the PLC-PCR show that the sensitivity of the reaction was 63.6% (95% CI: 52.9-74.4), NPV was 97% (95% CI: 95.8-98.1) and there was good agreement compared to the gold standard (Kappa value=0.8). Although targeting a single copy gene, PLC-PCR was able to significantly detect ~25 times more *T. brucei* s.l. infections than ITS-PCR (χ_1^2=44.5, p<0.001).

T. congolense species

Three sub species of *T. congolense* are recognised and can be detected using three different PCR reactions, each one specific for a different sub-species. These three sub-species are *T. congolense* Savannah, *T. congolense* Kilifi and *T. congolense* Forest, the respective specific PCR reactions for the diagnosis of these sub species are TCS, TCK and TCF.

The results in Table 5.3 show that specific PCR targeting *T. congolense* Savannah DNA diagnosed 79 animals to be infected. However, ITS-PCR identified only 21 animals to be infected with *T. congolense* species, the difference was statistically significant (χ_1^2=35.5, p<0.001). Only three animals were confirmed infected with *T. congolense* Savannah using both ITS-PCR and TCS-PCR reactions. Specific PCR for Kilifi and Forest types were negative in all the examined samples.

Using the gold standard defined as a positive result of ITS-PCR and/or species-specific PCR reactions for *T. congolense*, 97 animals were diagnosed to be infected with *T. congolense* species. Compared to the gold standard, the sensitivity of the specific PCR for *T. congolense* Savannah was 81.4% (95% CI: 73.7-89.2), NPV was 98% (95% CI: 97.1-98.9) and there was a very good agreement between the two reactions in identifying *T. congolense* (Kappa value=0.9). However, the sensitivity of ITS-PCR to identify *T. congolense* Savannah was 21.6% (95% CI: 13.5-29.8), NPV was 92% (95% CI: 90.3-93.7) and there was fair agreement when compared to the gold standard (Kappa value=0.3).

T. vivax species

T. vivax was identified in 41 animals using specific PCR reaction with the universal primers targeting the gene encoding *T. vivax* specific antigen, while ITS-PCR identified 24 animals, of which 10 were

confirmed by both tests, the difference between the two reactions was statistically significant (χ_1^2=4.6, p=0.03).

Interestingly, ITS-PCR identified 14 animals to be infected with *T. vivax* while they were not confirmed with the universal primers. Therefore, another set of primers targeting a highly repetitive G+C rich (64%) satellite DNA of 180 bp in size were used to investigate this disagreement between the universal TV-PCR and the ITS-PCR. However, the PCR reaction targeting the satellite sequence of *T. vivax* species was not able to identify any *T. vivax* isolate in the samples examined.

The gold standard for *T. vivax* identification was defined as any positive PCR result with ITS-PCR and/or TV-PCR for *T. vivax* species. The gold standard identified 55 animals to be infected with *T. vivax* species. The sensitivity of the universal TV-PCR compared to the gold standard was found to be 74.5% (95% CI: 63-86.1) with NPV of 98.5% (95% CI: 97.7-99.3) and a very good agreement (Kappa value=0.8). However, ITS-PCR sensitivity was only 43.6% (95% CI: 30.5-56.7) with 96.7% (95% CI: 95.6-97.9) NPV and good agreement compared to the gold standard (Kappa value=0.6) in the diagnosis of *T. vivax* species.

Other species

In addition to the aforementioned pathogenic trypanosome species, ITS-PCR was able to identify the non-pathogenic *T. theileri* species. The reaction diagnosed 46 animals to be infected with *T. theileri*. A species-specific PCR reaction that targets *T. theileri* satellite sequence of 500 bp size was developed by Rodrigues *et al*. (2003). This reaction was used in the current study but failed to amplify *T. theileri* DNA in the examined samples. Moreover, *T. simiae* was diagnosed by ITS-PCR in five animals, the species-specific reaction failed to amplify the examined samples. The lack of positive controls for *T. theileri* and *T. simiae* hindered the optimisation of these two reactions.

5.4.2 Breaking down the results according to sampling sites and animal species

Different animal species were used in the current study from Uganda and Zambia for the evaluation of ITS-PCR and species-specific PCR reactions. Further breakdown of the results was done according to the location of sample collection and the animal species.

5.4.2.1 Samples collected from Uganda

A total of 600 samples were collected from cattle in Uganda, DNA was eluted from 10 discs and the eluate was used for PCR reactions. Table 5.4 shows the results of ITS-PCR versus species-specific PCR reactions in cattle blood samples collected in Uganda.

Table 5.4: Number of cattle infected with trypanosomes diagnosed by different PCR reactions

	T. brucei s.l.	*T. congolense*	*T. vivax*	Total
ITS-PCR	1	13	17	31
Species-specific PCR	63	0	34	97

The results in Table 5.4 show that ITS-PCR identified 31 parasitic events, while species-specific PCR reactions identified 97 parasitic events, the difference was statistically significant (χ_1^2=38.1, p<0.001).

Looking at the individual species, ITS-PCR identified only one animal infected with *T. brucei* s.l. while, 63 animals were diagnosed with TBR-PCR, the difference between the two tests were statistically different (χ_1^2=63.4, p<0.001). The animal that was diagnosed infected with ITS-PCR was also confirmed to harbour *T. brucei* s.l. by TBR-PCR.

T. congolense was diagnosed using ITS-PCR in 13 animals, however, the species-specific PCR of the three *T. congolense* sub-species failed to amplify any of the samples.

T. vivax was identified in 17 animals using ITS-PCR, of which eight were confirmed using the universal primers for *T. vivax* species. TV-PCR identified 34 *T. vivax* infections in the examined cattle with a statistical significant difference between the two reactions (χ_1^2=5.9, p=0.02). Compared to a gold standard defined as any positive PCR result with ITS-PCR and/or TV-PCR for *T. vivax* species that identified 43 animals infected with *T. vivax*, the sensitivity of TV-PCR was 79.1% (95% CI: 66.9-91.2) with NPV 98.4% (95% CI: 97.4-99.4) and a very good agreement (Kappa value=0.9). However, ITS-PCR sensitivity was 39.5% (95% CI: 24.9-54.1) with NPV 95.5% (93.9-97.2) and a moderate agreement compared to the gold standard (Kappa value=0.5).

Finally, the non-pathogenic *T. theileri* species was identified in 46 cattle using ITS-PCR.

5.4.2.2 Samples collected from Zambia

A total of 369 samples collected from different animal species in Zambia in 2005 were kindly provided by Neil Anderson and Joseph Matemba. These samples were re-analysed using ITS-PCR and species-specific PCR reactions for trypanosome diagnosis using the same eluate from 10 discs. Table 5.5 shows the number of animals identified infected with trypanosomes using different PCR reactions.

Table 5.5: Number of animals sampled in Zambia infected with trypanosomes

Host	PCR	*T. brucei* s.l.	*T. congolense*	*T. vivax*	Total
Wildlife	ITS-PCR	1	7	7	15
	Species-specific PCR	3	47	4	54
Sheep	ITS-PCR	0	0	0	0
	Species-specific PCR	0	22	3	25
Dogs	ITS-PCR	0	1	0	1
	Species-specific PCR	11	10	0	21
Total	ITS-PCR	1	8	7	16
	Species-specific PCR	14	79	7	100

Compiled animal species

Parasitic events were identified in 16 animals using ITS-PCR, while 100 animals were diagnosed to be infected with trypanosomes using species-specific PCR reactions, the difference was statistically significant (χ_1^2=72.2, p<0.001).

ITS-PCR identified only one animal to be infected with *T. brucei* s.l. compared to 14 animals diagnosed with TBR-PCR, the difference was statistically significant (χ_1^2=11.5, p=0.001). The animal identified using ITS-PCR was also confirmed using TBR-PCR to have *T. brucei* s.l. infection.

T. congolense was identified in eight animals using ITS-PCR, of which three were confirmed to be *T. congolense* Savannah using TCS-PCR reaction. However, TCS-PCR identified 79 animals infected with *T. congolense* Savannah, the difference between the two reactions was statistically significant (χ_1^2=63.4, p<0.001). Using the gold standard defined as a positive result of ITS-PCR and/or species-specific PCR reactions for *T. congolense*, 84 animals were diagnosed to be infected with *T. congolense* species. Compared to the gold standard, the sensitivity of the specific PCR for *T. congolense* Savannah was 94.1% (95% CI: 89-99.1), NPV was 98.3% (95% CI: 96.8-99.8) and there was a very good agreement between the specific PCR reactions in identifying *T. congolense* and the gold standard (Kappa value=1). However, the sensitivity of ITS-PCR to identify *T. congolense* Savannah was 9.5% (95% CI: 3.2-15.8), NPV was 78.9% (95% CI: 74.7-83.2) and there was poor agreement when compared to the gold standard (Kappa value=0.1).

A gold standard defined as a positive result of ITS-PCR and/or species-specific PCR for *T. vivax* identified 12 animals infected with this species. ITS-PCR diagnosed seven *T. vivax* infected animals with only two confirmed positive using TV-PCR, also TV-PCR identified seven *T. vivax* animals with no statistical significant difference. The sensitivity of the ITS-PCR and universal TV-PCR compared to the gold standard was found to be 58.3% (95% CI: 30.4-86.2) with NPV of 98.6% (95% CI: 97.4-99.8) and good agreement (Kappa value=0.7), each.

T. theileri was not diagnosed in any of the samples collected from Zambia, while five animals were found infected with *T. simiae* using ITS-PCR.

Wildlife

Further breaking down of the results according to the species of the examined animals, Table 5.5 shows the number of wildlife infected with different trypanosomes using ITS-PCR and species-specific reactions. The results show that 15 parasitic events were detected in wildlife using ITS-PCR, while 54 were diagnosed with species-specific reactions, the difference was significant (χ_1^2=25.2, p<0.001).

Only one animal was found infected with *T. brucei* s.l. using ITS-PCR and it was also confirmed with the species-specific reaction, while three were diagnosed using TBR-PCR, the difference was statistically insignificant (p=0.6).

T. congolense was detected in seven animals using ITS-PCR, of which two were confirmed with the species-specific reaction of *T. congolense* Savannah. However, TCS-PCR diagnosed 47 animals to be infected with the species, the difference between the two reactions was statistically significant (χ_1^2=32.8, p<0.001). Using the gold standard defined as a positive result of ITS-PCR and/or species-specific PCR reactions for *T. congolense*, 52 animals were diagnosed to be infected with *T. congolense* species. Compared to the gold standard, the sensitivity of the specific PCR for *T. congolense* Savannah was 90.4% (95% CI: 82.4-98.4), NPV was 97.8% (95% CI: 95.9-99.7) and there was a very good agreement between the two reactions in identifying *T. congolense* (Kappa value=0.9). However, the sensitivity of ITS-PCR to identify *T. congolense* Savannah was 13.5% (95% CI: 4.2-22.7), NPV was 83.3% (95% CI: 78.8-87.7) and there was poor agreement when compared to the gold standard (Kappa value=0.2).

Regarding *T. vivax*, ITS-PCR diagnosed seven animals infected with the species and two of them were confirmed using the universal primers for *T. vivax*. Only four animals were found infected with *T. vivax* using the species-specific *T. vivax* PCR, the difference between the two reactions was statistically insignificant (χ_1^2=0.8, p=0.4). Compared to a gold standard defined as any positive PCR result with ITS-PCR and/or TV-PCR for *T. vivax* species that identified nine animals infected with *T. vivax*, the sensitivity of TV-PCR was 44.4% (95% CI: 12-76.9) with NPV 98.2% (95% CI: 96.6-99.8) and a good agreement (Kappa value=0.6). However, ITS-PCR sensitivity was 77.8% (95% CI: 50.6-100) with NPV 99.3% (95% CI: 98.2-100) and a very good agreement compared to the gold standard (Kappa value=0.9).

T. simiae was identified using ITS-PCR in five animals. However, none of the examined animals were shown to be infected with the non-pathogenic *T. theileri* species.

Sheep and Dogs

In sheep, ITS-PCR failed to identify any trypanosome infections, however, species-specific PCR diagnosed 25 parasitic events. *T. congolense* Savannah was detected in 22 animals while *T. vivax* was diagnosed in three animals.

A total of 21 dogs were found infected using the species-specific PCR, of which 11 were infected with *T. brucei* s.l. and 11 with *T. congolense* Savannah. However, ITS-PCR detected only one animal infected with *T. congolense* Savannah, this animal was also confirmed using the species-specific TCS-PCR.

5.4.3 Sequence analysis

The results from comparing the sensitivity of ITS-PCR amplification with other species-specific PCR reactions highlighted the need of further investigation into the specificity of the ITS-PCR reaction. Amplicons produced by this reaction were cloned in pDrive cloning vector (Qiagen) and sequenced (GATC). The sequences were analysed using DNASTAR software; the sequences were compared with reference sequences available on the NCBI database.

5.4.3.1 *T. brucei* s.l. sequencing

Two animal samples were identified by ITS-PCR to have *T. brucei* s.l. infections, one from a cow sampled in Uganda while the other was from an Impala sampled in Zambia. Also, bands obtained from ITS-PCR carried out on *T. brucei* s.l. isolate (BUT 135) were cloned and sequenced. BLAST searches were used to compare the *T. brucei* s.l. sequences obtained to reference sequences in the NCBI database. The closest matches for each sequence are summarised in Table 5.6.

Table 5.6: BLAST search results of *T. brucei* s.l. sequences

Sample ID	Origin of sample	Query coverage	Sequence length	Length of covered reference sequence	Closest match on NCBI BLAST	Percent identity to reference
Tb1	Cattle (Uganda)	100%	1210 bp	1216 bp	*T. brucei* NW2 18S ribosomal RNA gene (AF306776.1)	98%
					T. evansi 18S ribosomal RNA gene (D89527.1)	98%
Tb2	Wildlife (Zambia)	100%	1216 bp	1216 bp	*T. brucei* NW2 18S ribosomal RNA gene (AF306776.1)	98%
					T. evansi 18S ribosomal RNA gene (D89527.1)	97%
Tb3	BUT 135	100%	1219 bp	1219 bp	*T. brucei* NW2 18S ribosomal RNA gene (AF306776.1)	98%
					T. evansi 18S ribosomal RNA gene (D89527.1)	98%

The results in Table 5.6 show that *T. brucei* s.l. sequences from cattle, wildlife and BUT 135 were similar with 98% identity to the reference sequence of *T. brucei* s.l. ribosomal RNA sequence available in NCBI (Accession no. AF306776.1) and *T. evansi* 18S ribosomal RNA gene (D89527.1). Moreover, comparing the cattle and wildlife sequences revealed 98.4% identity of each other, while comparing BUT 135 to cattle and wildlife sequences revealed 97.9% and 97.6% identity, respectively. The expected band size of *T. brucei* s.l. from NCBI is 1207-1224 bp; the band size for *T. brucei* s.l. that was published by Cox *et al*. (2005) was 1215 bp but in the current study amplicon sizes of 1210 bp, 1216 bp and 1219 bp were obtained from cattle, wildlife and BUT 135 strain, respectively, which covers 1216 bp, 1216 bp and 1219 bp of the reference sequence, respectively.

5.4.3.2 *T. congolense* sequencing

Twenty one samples were identified to have *T. congolense* infection, 13 from cattle sampled in Uganda, seven wildlife samples and one dog sample from Zambia. The cattle samples were not identified with TCS-PCR, TCF-PCR and TCK-PCR, while three of the Zambia samples were confirmed with TCS-PCR. Six bands amplified from the Uganda samples and three bands amplified from the Zambia samples were successfully cloned and sequenced including one confirmed with TCS-PCR. Moreover, the amplified band from the *T. congolense* Savannah isolate Sikudo 124 which was isolated from a cow in Sikudo, Tororo district, Uganda, 1990 was cloned and sequenced. BLAST

searches were used to compare the *T. congolense* sequences obtained to reference sequences in the NCBI database. The closest matches for each sequence are summarised in Table 5.7.

Table 5.7: BLAST search results of *T. congolense* sequences

Sample ID	Origin of the sample	Sequence length	Query coverage	Length of covered reference sequence	Closest match on NCBI BLAST	Percent identity to reference
Tc1*	Cattle- Uganda	1132 bp	99%	1510 bp	*T. congolense* Forest (U22319.1)	93%
		976 bp	86%	1410 bp	*T. congolense* Savannah (U22315.1)	77%
Tc2	Cattle- Uganda	1446 bp	100%	1513 bp	*T. congolense* Forest (U22319.1)	92%
		1378 bp	95%	1413 bp	*T. congolense* Savannah (U22315.1)	78%
Tc3	Cattle- Uganda	1453 bp	100%	1513 bp	*T. congolense* Forest (U22319.1)	93%
		1410 bp	97%	1429 bp	*T. congolense* Savannah (U22315.1)	79%
Tc4*	Cattle- Uganda	1332 bp	100%	1513 bp	*T. congolense* Forest (U22319.1)	94%
		1255 bp	94%	1412 bp	*T. congolense* Savannah (U22315.1)	87%
Tc5	Cattle- Uganda	1461 bp	100%	1513 bp	*T. congolense* Forest (U22319.1)	93%
		1405 bp	96%	1418 bp	*T. congolense* Savannah (U22315.1)	79%
Tc6	Cattle- Uganda	1452 bp	100%	1513 bp	*T. congolense* Forest (U22319.1)	92%
		1409 bp	97%	1429 bp	*T. congolense* Savannah (U22315.1)	78%
Tc7*	Wildlife- Zambia	1184 bp	100%	1513 bp	*T. congolense* Forest (U22319.1)	93%
		848 bp	71%	1413 bp	*T. congolense* Savannah (U22315.1)	91%
Tc8	Wildlife- Zambia	1444 bp	100%	1513 bp	*T. congolense* Forest (U22319.1)	91%
		1131 bp	78%	1413 bp	*T. congolense* Savannah (U22315.1)	91%
Tc9**	Wildlife- Zambia	1391 bp	99%	1513 bp	*T. congolense* Forest (U22319.1)	78%
		1396 bp	100%	1413 bp	*T. congolense* Savannah (U22315.1)	95%
Tc10	Sikudo 124	1393 bp	76%	1413 bp	*T. congolense* Forest (U22319.1)	93%
		1145 bp	98%	1459 bp	*T. congolense* Savannah (U22315.1)	95%

***No overlap of the forward and reverse sequences **Sample confirmed with TCS-PCR**

The results in Table 5.7 show that all the sequenced amplicons from Uganda and Zambia are 91-94% similarity with *T. congolense* Forest (IL3900) isolate (U22319.1) and 77-91% similarity with *T. congolense* Savannah (IL1180) isolate (U22315.1). One sample that was confirmed as *T. congolense* Savannah with TCS-PCR showed 78% similarity to *T. congolense* Forest reference sequence and 95%

similarity to *T. congolense* Savannah reference sequence. Two sequences were 98-100% similar to *T. brucei* s.l. chromosome 2 reference sequence (AC012647.18), the two sequences were from wildlife and dog isolated in Zambia, the sequenced fragments were 210 and 496 bp in length, respectively. There is a variation in the size of the amplicons between different samples although all the sequences start and end with the ITS primer sequence. The band sizes of *T. congolense* Savannah and *T. congolense* Forest reported by Cox *et al*. (2005) are 1408 bp and 1501 bp, respectively.

5.4.3.3 *T. vivax* sequencing

ITS-PCR identified 24 animals infected with *T. vivax* species, of which 10 were confirmed with the *T. vivax* specific reaction. From Uganda, ITS-PCR identified 17 animals infected with *T. vivax*, of which eight were confirmed with the species-specific reaction. In Zambia, ITS-PCR identified seven wildlife samples infected with *T. vivax*, of which two were confirmed with TV-PCR. For sequencing, from the cattle samples collected in Uganda, eight samples identified with ITS-PCR were successfully cloned and sequenced of which one was confirmed with both ITS-PCR and TV-PCR. From the Zambia samples, four animals identified with ITS-PCR were successfully cloned and sequenced of which one was confirmed with TV-PCR. The BLAST search results of *T. vivax* sequences are summarised in Table 5.8.

Table 5.8: BLAST search results of *T. vivax* sequences

Sample ID	Origin of the sample	Sequence length	Query coverage	Length of covered reference sequence	Percent identity to reference*
Tv1	Cattle-Uganda	568 bp	100%	612 bp	87%
Tv2	Cattle-Uganda	570 bp	99%	612 bp	84%
Tv3	Cattle-Uganda	574 bp	98%	612 bp	86%
Tv4	Cattle-Uganda	573 bp	95%	612 bp	87%
Tv5	Cattle-Uganda	528 bp	100%	612 bp	86%
Tv6	Cattle-Uganda	554 bp	100%	612 bp	86%
Tv7	Cattle-Uganda	572 bp	100%	612 bp	85%
Tv8**	Cattle-Uganda	596 bp	100%	612 bp	94%
Tv9	Wildlife-Zambia	550 bp	100%	612 bp	86%
Tv10	Wildlife-Zambia	567 bp	96%	612 bp	85%
Tv11	Wildlife-Zambia	578 bp	95%	612 bp	83%
Tv12**	Wildlife-Zambia	587 bp	100%	612 bp	93%

***All the sequences were closely matched to *T. vivax* 18S, 5.8S, 28S and rRNA complete sequence (U22316.1) **Sample was confirmed with TV-PCR**

The results in Table 5.8 show that the sequenced amplicons from Uganda and Zambia are 83-87% similarity with *T. vivax* 18S, 5.8S, 28S and rRNA complete sequence (U22316.1), except the two

samples confirmed with TV-PCR were 93% and 94% similar to the reference sequence. The sequenced amplicon sizes were shorter than the 620 bp *T. vivax* band obtained by Cox *et al*. (2005), but they cover 612 bp expected sequence in the reference sequence.

5.4.3.4 *T. theileri* sequencing

T. theileri was identified in 46 animals sampled in Uganda; seven were successfully cloned and sequenced to validate the specificity of ITS-PCR reaction in the diagnosis of different trypanosome species. BLAST searches were used to compare the *T. theileri* sequences obtained to reference sequences in the NCBI database. The closest matches for each sequence are summarised in Table 5.9.

Table 5.9: BLAST search results of *T. theileri* sequences

Sample ID	Sequence length	Query coverage	Length of covered reference sequence	Percent identity to reference*
Tt1	964 bp	100%	991 bp	94%
Tt2	963 bp	100%	991 bp	95%
Tt3	949 bp	100%	991 bp	92%
Tt4	957 bp	100%	991 bp	89%
Tt5	952 bp	100%	991 bp	91%
Tt6	951 bp	100%	991 bp	91%
Tt7	934 bp	100%	991 bp	92%

***All samples are closely matched to *T. theileri* gene for 18S rRNA, 5.8S rRNA, 28S rRNA, partial and complete sequence (AB007814.1)**

The results in Table 5.9 show that all the sequenced amplicons from Uganda are 89-95% similarity to the *T. theileri* gene for 18S rRNA, 5.8S rRNA, 28S rRNA, partial and complete sequence (AB007814.1). The amplicon sizes were shorter than the 998 bp *T. theileri* band obtained by Cox *et al*. (2005), but the sequence covers 991 bp of the reference sequence on NCBI database.

5.5 Discussion

The aim of this work was to evaluate the pan-trypanosome ITS-PCR for the diagnosis of different trypanosome species compared to the species-specific PCR reactions. A total of 969 blood samples collected from different animal species in Uganda and Zambia were subjected to ITS-PCR and species-specific PCR reactions for the identification of different trypanosomes. Four species were identified with ITS-PCR including the three major pathogenic species; *T. brucei* s.l., *T. vivax* and *T. congolense*; and the non-pathogenic *T. theileri* species.

5.5.1 Comparing ITS-PCR with species-specific PCR reactions

ITS-PCR identified 47 pathogenic parasitic events compared to 197 diagnosed by the species-specific reactions, the difference was statistically significant.

T. brucei s.l. species

In the current study, ITS-PCR identified only two samples to have *T. brucei* s.l. infection; while the species-specific reaction diagnosed 77 parasitic events. The sensitivity of TBR-PCR was 100% compared to the gold standard that was defined as a positive result of ITS-PCR and/or TBR-PCR in amplifying *T. brucei* s.l. DNA. The low sensitivity of ITS-PCR in the diagnosis of *T. brucei* s.l. was reported by Auty (2009) who compared ITS-PCR and TBR-PCR for the diagnosis of *T. brucei* s.l. from blood samples collected from Tanzania on FTA®cards using DNA eluate. The sensitivity of ITS-PCR compared to TBR-PCR was 7% and the author attributed this finding to the low parasitemia of *T. brucei* s.l. in wildlife and the lower copy number of ITS target (100-200) in detecting *T. brucei* s.l when compared to the target amplified with the species-specific primers (10,000 copies).

This difference between the sensitivity of ITS-PCR and the species-specific PCR reactions might be related to the frequency of the target on the parasite genome. The copy number of ITS target in the genome is 100-200 compared to 10,000 and 5400 copies of satellite sequence specific for *T. brucei* s.l. and *T. congolense* Savannah, while *T. vivax* gene target was reported to be tandemly highly repeated. To investigate if the target copy number is a factor that determines the sensitivity of ITS-PCR in the diagnosis of trypanosomes; a PCR targeting the PLC single copy gene specific for *T. brucei* s.l. was compared to ITS-PCR. The results revealed that PLC-PCR was able to detect ~25 times more *T. brucei* s.l. infections than ITS-PCR. This suggests that it is not simply an issue of copy number.

In another study, ITS-PCR identified two *T. brucei* s.l. infected wildlife sampled in Zambia compared to 22 positive samples diagnosed by TBR-PCR (Anderson, 2009). In contrast, Njiru *et al*. (2005) reported that ITS-PCR using other primer sets were comparable to the species-specific reactions where ITS primers detected 84.9% of the samples positive using species-specific primers. The higher sensitivity of ITS primers compared with species-specific primers could be attributed to the sample processing method, because Njiru *et al*. (2005) used extracted DNA from blood samples with Qiagen extraction kits and 2 µl of the extract was used as a template in the PCR reaction. Also, Cox *et al*. (2005) evaluated ITS-PCR using the same primers used in the current study; they reported that ITS-PCR identified 40 samples positive for *T. brucei* s.l. while TBR-PCR identified 37 samples. These samples were collected on FTA®cards from cattle in Uganda; however, the PCR was conducted on the discs directly. There is no explanation of the discrepancy between the results of Cox *et al*. (2005) and the current study regarding the sensitivity of ITS-PCR in the detection of *T. brucei* s.l. DNA. There is no reported data from Cox *et al*. (2005) study regarding the number of discs examined and the age of the samples that could have been a factor to explain such differences.

T. congolense species

It was difficult to differentiate between the three sub-species of *T. congolense* during the visualisation of the amplified targets using the UV transilluminator, even after prolonged separation time of the

bands by electrophoresis. This is attributed to that the band sizes of the amplified products of the three types are close to each other (1513 bp, 1422 bp and 1413 bp for *T. congolense* Forest, *T. congolense* Kilifi and *T. congolense* Savannah, respectively). For this reason, any product with a band size above 1400 bp was classified as *T. congolense* species in the current study. The PCR reactions specific for *T. congolense* Forest and *T. congolense* Kilifi were negative for all the examined samples.

A total of 79 animals were diagnosed infected using TCS-PCR, while only 21 were categorised as *T. congolense* species using ITS-PCR. The sensitivity of ITS-PCR and TCS-PCR compared to a gold standard which in this case defined as any positive PCR result with ITS-PCR and/or TCS-PCR for *T. congolense* species was 81.4% and 21.6%, respectively. Only three samples positive for *T. congolense* with ITS-PCR were confirmed with the species-specific PCR for *T. congolense* Savannah. The inability of TCS-PCR to confirm 18 samples diagnosed with ITS-PCR were surprising because it is assumed that TCS-PCR would identify more *T. congolense* Savannah species due to the higher copy number of the target in the trypanosome genome (5400 copies). The possibility of these samples to be related to *T. congolense* Forest or *T. congolense* Kilifi was excluded due to the negative TCF-PCR and TCK-PCR results. In order to clarify the identity of those species diagnosed as *T. congolense* by ITS-PCR, cloning and sequencing was done and the results of the sequencing is discussed later in this chapter (section 5.5.3).

Cox *et al*. (2005) compared ITS-PCR with species-specific reactions on cattle blood samples collected on FTA®cards from Uganda. ITS-PCR identified six samples as *T. congolense* while only one sample was diagnosed using specific reactions for *T. congolense* Savannah. These findings do not coincide with the current study and there is not enough information to discuss the possible reason for this discrepancy.

T. vivax species

In the current study, universal primers targeting the gene encoding *T. vivax* specific antigen were used for the diagnosis of *T. vivax* species. The universal primer set diagnosed 41 *T. vivax* infections while ITS-PCR identified 24 positive samples. The sensitivity of the universal TV-PCR and ITS-PCR were 74.5% and 43.6%, respectively, compared to the gold standard that was defined as any positive PCR result with ITS-PCR and/or TV-PCR for *T. vivax* species. The higher sensitivity of TV-PCR could be related to the copy number of target which is reported to be tandomly in the trypanosome genome. Fourteen samples were identified as *T. vivax* with ITS-PCR but they were negative using the universal *T. vivax* primers. Therefore, another set of primers targeting a satellite DNA sequence were used and none of the samples were positive with these primers. This was not unexpected because the primers targeting the satellite sequence are not present in some *T. vivax* isolates (Masake *et al.*, 1997). Cox *et al.* (2005) identified nine *T. vivax* infections in cattle blood samples using the primer set amplifying the satellite sequence, while ITS-PCR identified six *T. vivax* infections.

Fourteen samples were diagnosed positive for *T. vivax* by ITS-PCR while they were not confirmed with TV-PCR; therefore cloning and sequencing of PCR products was done to clarify the identity of these samples. The discussion of the sequencing results are detailed later in this chapter (section 5.5.3).

5.5.2 Sequence analysis

The specificity of ITS-PCR was evaluated by cloning and sequencing a number of the amplified products of the second round representing the different trypanosome species.

5.5.2.1 *T. brucei* s.l. sequencing

T. brucei s.l. was only identified in two samples, one from a cow in Uganda and the other from an Impala in Zambia. The amplified products from these two samples and from *T. b. brucei* procyclic form (BUT 135) were cloned and sequenced; the sequences were compared to the reference sequences in the NCBI database. The three amplicons were 98% identical to *T. brucei* NW2 18S rRNA gene (AF306776.1). According to information available in PubMED the reference strain was isolated in 1992 from a human in Uganda and was classified as *T. b. gambiense*. Identity of the sequenced amplicons with other reference *T. brucei* s.l. ranged from 95-97%. The other reference sequences available included *T. b. gambiense* isolated from a human in Cote d'Ivoir and Sudan, also *T. b. brucei* isolated from a pig in Nigeria, a fly in Cote d'Ivoir and a lion in Tanzania. Moreover, the sequences in the current study were 96-98% identical to different *T. evansi* isolates from different host species (cattle, buffalo and deer) isolated from Thailand and Taiwan. This indicates low variation in *T. brucei* s.l. and *T. evansi* species in different areas and hosts. This conclusion was supported by Cortez *et al*. (2006) who reported more conserved ITS sequences within *T. b. brucei* isolates, although 2.8% and 1.2% polymorphism in ITS1 and ITS2 sequences, respectively, exists. Also, these results indicate that ITS-PCR is specific for *Trpanozoon* species despite the lower sensitivity of this reaction in amplifying *Trypanozoon* DNA. The similarity between *T. brucei* s.l. and *T. evansi* reference sequences was 98.9%.

Similar results were obtained by Auty (2009) who sequenced *T. brucei* s.l. products identified in hyaena and zebra from Tanzania; the sequences were >97% identity with the reference sequences. The band sizes obtained in the current study were 1210 bp, 1216 bp, 1219 bp in the cattle, Impala and BUT 135 isolates, respectively. The obtained amplicon sizes are within the expected band size from NCBI database (1207~1224 bp), while they were longer than the band size of 1215 bp obtained by Cox *et al*. (2005). Auty (2009) obtained 1220 bp and 1207 bp from the hyaena and zebra isolates, respectively. These variations could be a result from sequencing or cloning errors which might result in adding or missing of some bases.

5.5.2.2 *T. congolense* sequencing

ITS-PCR identified 13 cattle samples collected in Uganda and eight samples collected from Zambia to have *T. congolense* infections; it was difficult to differentiate between the three *T. congolense* types by visualisation of the bands after gel electrophoresis. Therefore, bands of sizes more than 1400 bp were classified as *T. congolense* species. The species-specific PCR for the three *T. congolense* types were unable to identify any of the *T. congolense* species diagnosed from cattle by ITS-PCR, while three isolates from Zambia were confirmed with TCS-PCR. To clarify the identity of these isolates, six amplified products from cattle samples and three from Zambia collected samples were successfully cloned and sequenced. Moreover, the amplicon obtained from the ITS-PCR of Sikudo 124 isolate procyclic form was also cloned and sequenced.

The BLAST search of the *T. congolense* isolates from cattle in Uganda resulted in 92-94% identity with *T. congolense* Forest (IL3900, U22319.1) that was isolated from a dog in Burkina Faso, and 77-87% identity with *T. congolense* Savannah (IL1180, U22315.1) that was isolated from Tanzania. The similarity of *T. congolense* Forest reference sequence (IL3900, U22319.1) and *T. congolense* Savannah reference sequence (IL1180, U22315.1) is 73.5% and the alignment of the two sequences is illustrated in Figure 5.3.

```
T.c.forest.ref        GGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGCTCATTTTCCGAT 60
T.c.savannah.ref      GGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGCTCATTTTCCGAT 60
                      ************************************************************

T.c.forest.ref        GATAAAAAA-GTATACATACATATGTGTAC-GTGTA-GTGTAGGTGTGTGCTATCGAAGG
117
T.c.savannah.ref      GATAATATATATATACATATGCGTGTATATTATACGCATGTGGGTGTTTGTTGTGAGAGG
120
                      ***** * *  ********    *** **   *     *** ***** ** * *   ***

T.c.forest.ref        TTGTTGTTGT---GTGCTTGTGTGC---------CCCTCGCTCATGCG---CATCCCCAT
162
T.c.savannah.ref      TTGTTGTTGTTGTGTGCTCGTGTGCGTACGGTGCCCCTCGTTCGTGCGAATTATTCCCAT
180
                      **********   ***** ******         ****** ** ****    ** *****

T.c.forest.ref        CC-CGCACGCCCCAGTGTTTTGTGTGCTGTGCGATGCGGTGTGTGTTGGGTCGCGCATTG
221
T.c.savannah.ref      CCGCATCCGCCCCGGTGTGGTGTGCGGTGTGTGTTGGGG--------GAGCCGCACGTGG
232
                      ** *   ****** ****  **** * **** * ** **        * * *** * * *

T.c.forest.ref        TCGG-CGCTGT-GATGTGCCGGCCACGAACCTTGAAAACCTTGAAGCACGTCTCGCGAAA
279
T.c.savannah.ref      TGGGGTGCTGCCGTTGTACCGGCCGC---------AATCTCTAAAACGCGCCTCGGAGCA
283
                      * **  ****  * *** ****** *         ** *  * ** * ** ****    *

T.c.forest.ref        CACACGTGTCCAAGCACGCGTCTCC-ATGTCGCTGTCTCTCTCTTGTGTTGCGAAGATGC
338
T.c.savannah.ref      CGCACGTGTCCAAACACGCGTCCCCCATGTCGCTCTCTTTCTCTTGTGTTGCGAGGGTGC
343
                      * *********** ******** ** ******** *** *************** * ***

T.c.forest.ref        TTACTGTCGTGTTTGCTCCGCCAGGCGGCGCGGCCGTGACGTATGTGTCCGCAAAGACAA
398
T.c.savannah.ref      TTACGGTTGTGTGCGCGCC--------------CCGCAAGG---------GCAAGGAAGA
380
                      **** ** ****  ** **              ***  * *         **** **  *
```

```
T.c.forest.ref        AAGAAG-GGTGTGGAGGAGACGGCGTGTTCTTATGCCGCCTGACGCTTTTTGTGTGCGCA
457
T.c.savannah.ref      AGGAGGTGGTGTGGAGGAGACGACGTGTTCTTATGCCGCCCGACGCTTATTGTGTGCGCA
440
                      * ** * *************** ***************** ******* ***********

T.c.forest.ref        CCGGCTCGGCTCACTTTTTCCTCCCTCTCCTCTTTTCCTCCTCTTCTTTTCCCCGCCTTC
517
T.c.savannah.ref      CTGGCTCGCTT---TTCTCCCTCTTCTTCTCCTCCTCGTCCTCATCTTTTCCAAGCCTTC
497
                      * ******  *   ** * ****    **  **  ** ***** ********  ******

T.c.forest.ref        CCACGTGTGTTGGGAGAGGACAGAGGGCAAGAAGGCGTGGTGTATTTGGAGGAAT---GT
574
T.c.savannah.ref      CCACGTGTGTTGGGAGAGT-----GGAAGAGGAAGTGTG-TGTGTTTGGAGGAAGAAGGT
551
                      ******************      **   ** * * *** *** **********    **

T.c.forest.ref        GCGGGGTGAAA----TGGTGAAAGCGTGCGTGCGTGCGCAGGCGT--TGGTCACGGCTCT
628
T.c.savannah.ref      GCAGTGGGAGAAATATGGTGAGTGCTTGTGTGTGTACGCAGGTGTGTTGGTCACGGCTCT
611
                      ** * * ** *    ******  ** ** *** ** ****** **  **************

T.c.forest.ref        CACAACGTGTCGCGATGGATGACTTGGCTTCCTATTTCGTTGAAGAACGCAGCAAAGTGC
688
T.c.savannah.ref      CACAACGTGTCGCGATGGATGACTTGGCTTCCTATTTCGTTGAAGAACGCAGCAAAGTGC
671
                      ************************************************************

T.c.forest.ref        GATAAGTGGTATCAATTGCAGAATCATCACATTGCCCAATCTTTGAGCGCAAACGGCGCA
748
T.c.savannah.ref      GATAAGTGGTATCAATTGCAGAATCATCACATTGCCCAATCTTTGAACGCAAACGGCGCA
731
                      ********************************************** **************

T.c.forest.ref        TGGGAGAAGCTCTTCCGAGCCATCCCCGTGCATGCCACATTCTCAGTGTCGACCAACATA
808
T.c.savannah.ref      TGGGAGAAGCTCTTCCGAGTCATCCCCGTGCATGCCACATTCTCAGTGTCGACCAACAAA
791
                      ******************* ************************************ *

T.c.forest.ref        AGAACAACAACAGTGCCCTCTTCCCTCTGTTTCTCTCTCTGGTGATGGGCAGGGTGTGGG
868
T.c.savannah.ref      AAAACAACAGCAG--CCCTCTTCT---TCTCCCTGTCTCTGATGACGAGCATGGTGTGTG
846
                      * ******* ***  ********    * *  ** ****** *** * *** ****** *

T.c.forest.ref        GTGTGTGCTGTGTATATGTTGTTGTCGTGGCGTGTGTGAAAGTGGTGTGGTGGGGCGACG
928
T.c.savannah.ref      GTATGTG-TGTGC-TGTAT-GTTCTCGTGACGTGCTTGAAAATGGGGCGG---------G
894
                      ** **** ****  * * * *** ***** ****  ***** *** * **         *

T.c.forest.ref        CGCATGTGCGCATCGTTGTTACGTACGTACATTTACGCACACACACGTGTGCTGCGTGTG
988
T.c.savannah.ref      CGCGTGTATGTACCGTCGTC----------------------------------------
914
                      *** ***  * * *** **

T.c.forest.ref        TATGTGTTTGTTGTCCCCATTATTATTTTTCATGCCGTCAGGAAGGAGAGAGTCCGGTGT
1048
T.c.savannah.ref      --------------CCCTATTAATATT--TCATGCCGTCAGGAGGGAGAGAGTCCGGTGT
958
                                 *** **** ****  ************** ****************

T.c.forest.ref        GTGCATAGTGGTGTTGTTGCG-TGCGTGTGAAGGGTTGTGTTTGTGTTCTATATGAGTGT
1107
T.c.savannah.ref      GTGTATTGTGGTGTTGTTACGACGTGTGAGAAGGGTTGTATGTGGTATGTGTGTGGAGGT
1018
                      *** ** ************ **  * *** ********** * **   * * * **   **
```

```
T.c.forest.ref        GTGGCATGTGTGA-GTGGTGTCTGTGCAAGAAGGTGTGGTGTCCTTGTGGGCGTGCTTTT
1166
T.c.savannah.ref      GTGGAGTCTGTGGCACGGGGGCTGTG--------TGTGGTGTCTTTGTGGGCG-GCGTGC
1069
                      ****  * ****    ** * *****        ********* ********* ** *

T.c.forest.ref        GCATGTTGTTTGTTCCCGCTTGAGAC--GGCCCCGCTTTTCTCCCCTCACTTTCTCTCCC
1224
T.c.savannah.ref      TCTTGC-ATTTGTTCCC-CTTGAGACACGACCCCTCTCTCGTCTCCTT-CTTTCCCTCTC
1126
                       * **   ********* ********  * **** ** *  ** ***  ***** *** *

T.c.forest.ref        CGTGCTGCCATCACTACACTTCCTTTCGGCCATA--GCAGTAGAAC---ACCCTTGCCTC
1279
T.c.savannah.ref      CGCGTCATCATCGC--CGCTTCCTTTCACCCGTGTCGCAGTAGAACGCCACCTTTACCTC
1184
                      ** *    **** *  * *********  ** *   **********   *** ** ****

T.c.forest.ref        CCCCGGTGTGTTTACCTCGAAGCCATTTGAGCTAAAGAGACAAAGTGGGGA-ATGATCTT
1338
T.c.savannah.ref      CTCCGGTGT--TTACCTCGAAGCTATTTGAGCTAAAGAGACAAAGTGGGGATGTTGCTTG
1242
                      * *******  ************ ***************************  *    *

T.c.forest.ref        GGAGAGAGGCTCTTGTCTTCTCTCACCTCCACATAACTGTTGTGCTTTGTGCATCCCGT-
1397
T.c.savannah.ref      GGAGGGAGGCTTTCTTCCTTCCTCAGCAGTCCTCACCC----CGCATTGCGGGACGGGTG
1298
                      **** ****** *  ** *  **** *    *  * *      ** *** *   *  **

T.c.forest.ref        CACGCGATGTACGCACGCTAACCAAAGTTCATATGTATGTACATGTTGACAGACCTGAGT
1457
T.c.savannah.ref      TGTGCG-TGAGCGCACATCTGCAAGAATTTATATATGTATACATGTTGACAGACCTGAGT
1357
                         *** **  *****     * * * ** **** * * **********************

T.c.forest.ref        GTGGCAGGACCACCCGCTAAACTTAAGCATATTACTCAGCGGAGGAAAAGAAAACAA 1514
T.c.savannah.ref      GTGGCAGGACCACCCGCTAAACTTAAGCATATTACTCAGCGGAGGAAAAGAAAACAA 1414
                      ************************************************************
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Figure 5.3: Alignment of *T. congolense* Forest (IL3900, U22319.1) and *T. congolense* Savannah (IL1180, U22315.1) reference sequences (forward and reverse primers are red in color, *shows the similarity between the two sequences)

The samples that were not confirmed with TCS-PCR or TCF-PCR were 91-94% similarity to *T. congolense* Forest, while showing 77-91% similarity to *T. congolense* Savannah. This resulted in inability to accurately classify these samples especially with 73.5% similarity of *T. congolense* Forest and *T. congolense* Savannah reference sequences. Therefore, these samples were classified as *T. congolense* species.

Looking at sequence of the sample that was confirmed with TCS-PCR and the *T. congolense* Savannah isolate (Sikudo), both were 95% similar to *T. congolense* Savannah IL1180, while 78% and 93% identical to *T. congolense* Forest IL3900, respectively. Despite these samples were identified as *T. congolense* Savannah but the close similarity to *T. congolense* Forest made it difficult to classify to the sub-species level using ITS-PCR. Auty (2009) diagnosed *T. congolense* species from wildlife in Tanzania and by sequencing; the sample was 92% similar to *T. congolense* Savannah isolate (IL1180).

The difficulty to differentiate *T. congolense* Savannah and *T. congolense* Forest using the ITS target was reported using different primer sets targeting the ITS region. Kin primers designed by Desquences (1997) showed only 30 bp difference between the two sub-species, while only 3 bp differences was reported using the primers designed by Geysen *et al*. (2003). Also, ITS-CF and ITS-BR primers do not differentiate between *T. congolense* Savannah and *T. congolense* Forest with 700 bp product, each (Njiru *et al.*, 2005). Moreover, ITS1 primers designed by Adams *et al*. (2006) do not differentiate between the two sub-species with 640 bp amplicon, for each.

The inability of the species-specific PCR reactions to identify the *T. congolense* species diagnosed with ITS-PCR could be explained by the existence of a new *T. congolense* sub-species which is similar to *T. congolense* Savannah and *T. congolense* Forest in ITS target, while there is no specific reaction to identify such isolate. Moreover, variation within *T. congolense* Savannah isolates was confirmed by Cortez *et al*. (2006) who reported a high polymorphism of ITS sequences (9.7% for ITS1 and 8.6% for ITS2) within *T. congolense* Savannah. Moreover, isoenzyme analysis by Gashumba *et al*. (1998) showed that *T. congolense* from Uganda was separated from other *T. congolense* isolates suggesting that further analysis of *T. congolense* isolates may be required.

In conclusion, it was decided to classify any of the isolates with band size more than 1400 bp as *T. congolense* species regardless to the sub-species classification. In the other chapters in the current study it was decided to use TCS-PCR for the diagnosis of *T. congolense* Savannah because the sensitivity of this reaction was 81.4%, while ITS-PCR sensitivity was 21.6% in identifying *T. congolense* species.

5.5.2.3 *T. vivax* sequencing

Eight isolates identified with ITS-PCR as *T. vivax* sampled from cattle in Uganda of which only one was confirmed with TV-PCR and four isolates from wildlife in Zambia diagnosed as *T. vivax* with ITS-PCR of which one was confirmed with TV-PCR were successfully cloned and sequenced to clarify the identity of the samples. All the sequenced samples identified only with ITS-PCR showed 83-87% identity with *T. vivax* ILDat1.2 (U22316.1) reference sequence with 100% coverage of the sequence starting and ending from the designed primers, the origin of such isolate is Nigeria (West Africa). Also, the sequences showed 87% identity with different *T. vivax* isolates from Brazil but with only 69% sequence coverage. Moreover, the sequences in the current study were 88% similar to an isolate from a cow in Kenya (IL3905) with 56% coverage of the sequence due to the availability of only partial sequence of the ITS1 region on the NCBI database.The two isolates that were confirmed with TV-PCR showed 93% and 94% similarity to the reference sequence of the isolate from Nigeria while 88% similarity to the East African isolate from Kenya. The existence of similarity between the isolates in the current study and the African isolates (East and West African isolates) was expected, this was supported by Gardiners (1989) who reported that *T. vivax* from Central Africa (Uganda and Congo) shared molecular features with both the East and West African isolates.

Phylogenetic analysis indicated that *T. vivax* from West Africa and South America are very similar, while the isolates from East Africa are more diverse (Cortez *et al.*, 2006). This variation in *T. vivax* sequence is expected due to the reported genetic variants of *T. vivax* that exists in East Africa that could be attributed to multiple route of transmission because *T. vivax* could be transmitted cyclically and mechanically by biting insects, compared to consistent sequences in West Africa and South America where only mechanical transmission occurs (Rodrigues *et al.*, 2008). These findings were supported by Njiru *et al*. (2005) who reported the existence of genetic variants for *T. vivax* in Kenya, also Masake *et al*. (1997) and Malele *et al*. (2003) reported *T. vivax* genetic variants in the field. Evidence of size variation in ITS sequences within species were reported, for example, the length of ITS1, 5.8S and ITS2 regions in *T. vivax* from South America and West Africa was 490 bp, while *T. vivax* from East Africa were longer varying between 525 bp and 534 bp and also varied between gene copies of the same isolate (Cortez *et al.*, 2006). The inability of the *T. vivax* specific primers in the current study to confirm some of the isolates identified with ITS-PCR could be attributed to the existence of genetic variants of *T. vivax*.

Similar results were obtained by Auty (2009) who showed 81% similarity of *T. vivax* isolated from a giraffe in Tanzania with the reference *T. vivax* sequence ILDat1.2 (U22316.1), while 97% and 89% similarity of another two isolates from Buffalo and waterbuck, respectively, to IL3905 isolate from Kenya. The authors referred to the isolates as *T. vivax*-like because they shared only 80-90% identity with any *T. vivax* reference sequence.

Gonzales *et al*. (2006) reported that the sensitivity of the ITS primers in the diagnosis of *T. vivax* was the lowest despite using different sample preparations compared to species-specific primers. They attributed the lower sensitivity of these primers to the partial homology of the ITS1 sequences and their limited repetitiveness.

5.5.2.4 *T. theileri* sequencing

A total of 46 cattle were diagnosed to have *T. theileri* infections, while none of the samples collected in Zambia were identified as *T. theileri*. Seven amplified products were successfully cloned and sequenced to validate the specificity of ITS-PCR. The obtained amplicons showed 89-95% similarity to the available *T. theileri* sequence on NCBI website that is isolated from Brazil. The geographic difference would explain the divergence between the current isolates and those located in NCBI database where there is no available sequences representing the African isolates. The existence of divergence between the South American and African isolates could be supported by the inability of the species-specific PCR for *T. theileri* to amplify any of the samples identified with ITS-PCR.

5.5.3 Conclusion

In conclusion, the development of treatment and control strategies to protect livestock and humans from trypanosomiasis requires accurate data regarding the disease which in turn depends on accurate diagnosis and definitive identification of the causative species (Thumbi *et al.*, 2008). Parasitological and serological methods are not reliable due to low sensitivity and specificity, respectively, while accurate detection of trypanosomes in both the host and vector heavily depends on PCR. The performance of PCR depends on the sample preparation, DNA extraction method and the primers used (Gonzales *et al.*, 2006).

ITS primers are used for the identification of different trypanosomes using a nested reaction, however, questions regarding the sensitivity and specificity of the primer set rose during this study. Despite being specific to *Trypanozoon*, ITS primers showed low sensitivity compared to the species-specific reaction. This was firstly thought to be attributed to the higher copy number of the specific PCR target but this was not the case. ITS-PCR is unable to differentiate between *T. congolense* sub-species and also showed low sensitivity compared to the specific reaction for *T. congolense* Savannah. However, ITS-PCR sensitivity in the diagnosis of *T. vivax* was found 43.6% compared to 74.5% sensitivity of TV-PCR. The issue of ITS-PCR lower sensitivity especially with *T. brucei* s.l. required the use of the specific primers in the current study. The study conducted by Auty (2009) also recommended the use of specific-species primers instead of the ITS primers designed by Cox *et al*. (2005) when studying trypanosomes prevalence in wildlife. This was due to the low sensitivity of ITS primers in amplifying *T. brucei* s.l. and the inability to differentiate *T. congolense* and *Trypanozoon* sub-species.

For studying prevalence data, species-specific primers are more suitable in terms of sensitivity, while for studying diversity of trypanosome species ITS primers could be used and accompanied by sequencing of the obtained products for interpretation of the obtained results (Auty, 2009). However, other primers targeting ITS region could be designed to increase the sensitivity of the reaction and to differentiate between different *T. congolense* sub-species because the requirement of a pan-trypanosome reaction would greatly reduce the cost and time in processing samples.

6 Chapter six:

Case study one: Molecular approaches in studying the impact of the SOS campaign on different trypanosome species, Uganda

6.1 Introduction

Tsetse transmitted trypanosomes affect nearly nine million km^2 of sub-Saharan Africa, precluding the keeping of livestock in areas of high challenge; leading to disruption in the development of sustainable mixed farming over large and potentially productive areas (Hursey, 2001). Trypanosomiasis, a disease that tsetse flies transmit has been endemic in south-eastern and north-western Uganda for two decades (Wendo, 2002) where it is known to be a severe constraint to human and animal health. Moreover, it has a great impact on the productivity of animals kept in endemic areas, where the disease sometimes rises to epidemic levels (Waiswa and Katunguka-Rwakishaya, 2004).

In Uganda, it is estimated that *Glossina* species infest 106,400 km^2 (50% of the entire landmass) leading to exposure of more than 40% of cattle to trypanosome infection risk (Magona *et al*., 2004). According to a survey carried out in late 2001, 8% of the cattle in south-eastern Uganda have the microscopic blood-borne parasites but many of them do not show symptoms (Wendo, 2002).

Proper diagnosis of the disease using molecular tools is important for the intervention decisions and to follow up the campaign for rational evaluation of the success or failure of such programme. The results in chapters IV and V concluded that for the diagnosis of trypanosome species, genomic material of an appropriate quality and concentration are required. Elution of trypanosome DNA from FTA®cards, containing blood samples examined directly after collection, (10 discs) using Chelex®100 obtained genomic material of sufficient quality and quantity overcoming the uneven distribution of the genomic material on the card matrix. Specific targets are required for accurate diagnosis of trypanosomes; the study recommended that using species-specific PCR reactions would give an acceptable level of accuracy about the disease prevalence due to higher sensitivity of the specific reactions compared to ITS-PCR.

The spread of *T. b. rhodesiense* into previously unaffected districts in Uganda, including Kabermaido, Lira and Dokolo, Uganda, was reported in August 2004 (Wissmann *et al.*, June, 2007). The main cause for such spread was suspected to be the movement of cattle from established animals trypanosomiasis and Human African Trypanosomiasis (HAT) endemic areas northwards into the newly affected districts (Fevre *et al.*, 2001; Kabasa, 2007). The newly introduced animals bring with them the human infective *T. b. rhodesiense* as well as the trypanosome species causing animal Nagana, to areas previously free of human and animal infective parasites (Kabasa, 2007).

The World Health Organization (WHO) recommended the block treatment of cattle using trypanocidal drugs at a meeting conveyed in 2005. In response, the Stamp Out Sleeping sickness campaign (SOS) was initiated in late 2006 in central Uganda by the University of Edinburgh and Makerere University,

Uganda, in partnership with CEVA Santé Animale (a French based pharmaceutical veterinary laboratory) creating a public private partnership. The campaign was funded by Industri Kapital which is a European private equity firm (Kabasa, 2007).

6.2 Objectives

The aim of the current chapter is to evaluate and follow up the impact of a treatment programme conducted in central Uganda on trypanosome species using DNA eluted from 10 whole blood discs to amplify the species-specific targets with PCR reactions.

6.3 Material and methods

6.3.1 Stamp Out Sleeping sickness campaign

The aim of phase I of SOS campaign was the mass treatment of the cattle population in the newly epidemic areas with trypanocidal drugs followed by prophylaxis from re-infection through application of insecticides by the spraying of cattle (hence using them as live-bait). The treatment of the animal reservoirs is more beneficial in terms of prevention of new cases of sleeping sickness arising, as it is estimated that cattle-tsetse-human transmission cycle for *T. b. rhodesiense* is five times more likely to occur than human-tsetse-human cycle (Hide, 1999; Hide *et al.*, 1996; WHO, 2006).

The impact of the mass treatment programme on the cattle reservoir was monitored by repeated cross-sectional sampling in 23 sentinel villages distributed across the project area. In each village, 80-100 cattle were monitored at baseline (immediately before mass-treatment in November, 2006), 3 months (January, 2007) and 9 months (July, 2007) post-implementation of the mass treatment with blood samples being analysed by PCR.

In July, 2006, a survey was carried out in the study area to obtain an estimation of the prevalence of *T. brucei* s.l. to decide the type of trypanocide to be used in each area. There were insufficient stocks of one trypanocide to treat the whole area, therefore, in areas of high transmission, isometamedium chloride (ISM, Veridium) was used due to prophylactic period of two to three months provided by ISM to decrease re-infection rate. Diminazine-aceturate (DI) was used in areas with low prevalence. The trypanocidal drug used to treat cattle from Kabermaido and Dokolo was ISM with a dose 1 mg/kg body weight. While DI was used in a dose 7 mg/kg, it has a curative effect without any prophylactic effects. All cattle were sprayed with deltamethrin insecticide (Vectocid) using the restricted application technique to the tsetse feeding sites (legs and belly) to control the vector aiming to reduce the re-infection (Kabasa, 2007). The restricted application of insecticides to the belly and legs with two weeks intervals was found to be more cost-effective rather than treating the whole body of the animal at monthly intervals. This would cut the cost of insecticides by 40% and improve efficacy by 27% (Torr *et al.*, 2007).

6.3.2 Study area

The project was conducted in five districts in Uganda, these were, Kabermaido, Dokolo, Lira, Amolatar and Apac. In the current work, three districts (Kabermaido, Dokolo and Lira) were chosen because they are new foci for sleeping sickness and due to time constraints in analysing the samples collected from the five districts. Four villages were randomly selected from each district at three time points (baseline, three months and nine months) for analysis during this study. Figure 6.1 shows the districts included in the project, Figure 6.2 shows the distribution of the 23 sentinel villages included in the project and the 12 villages selected for the current work.

6.3.2.1 Kabermaido district

Until 2000, Kabermaido district was part of Soroti district. It borders Soroti in the east, Lira in the west, Kamuli in the south and Katakwi in the north-east. The district lies in 1,627.9 km^2 at an approximate altitude of 1036 m-1127 m above sea level, with rainfall 1000-1500 mm per annum with high temperatures.

6.3.2.2 Lira district

This district lies in northern Uganda and borders Apac in the west, Kabermaido in the south-east, Katakwi in the east and Pader in the north. It lies in 7200.7 km^2 at an approximate altitude of 975 m-1146 m above sea level. The rainfall ranges between 1000-1500 mm with high temperatures.

6.3.2.3 Dokolo district

Until July, 2006, Dokolo district was one of the counties of Lira district. It borders Lira in the north, Kabermaido in the east and southeast, Amolatar in the south and Apac in the west. The rain fall ranges between 1000-1500 mm and is accompanied by high temperature.

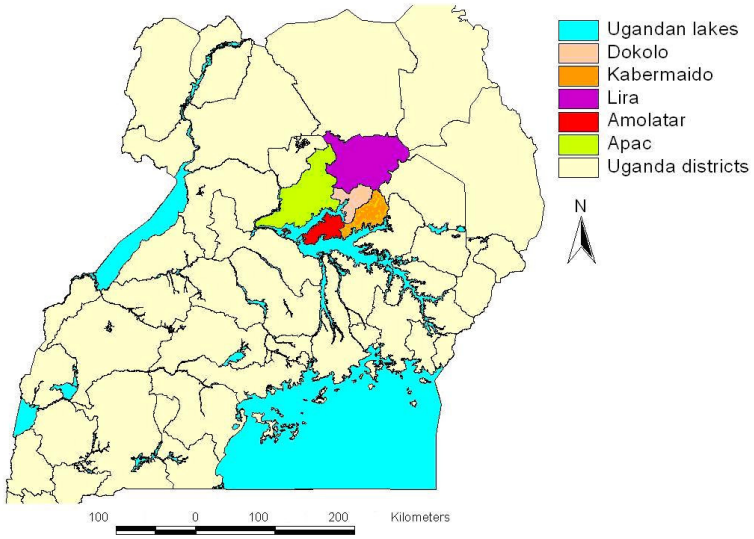

Figure 6.1: SOS project districts, central Uganda

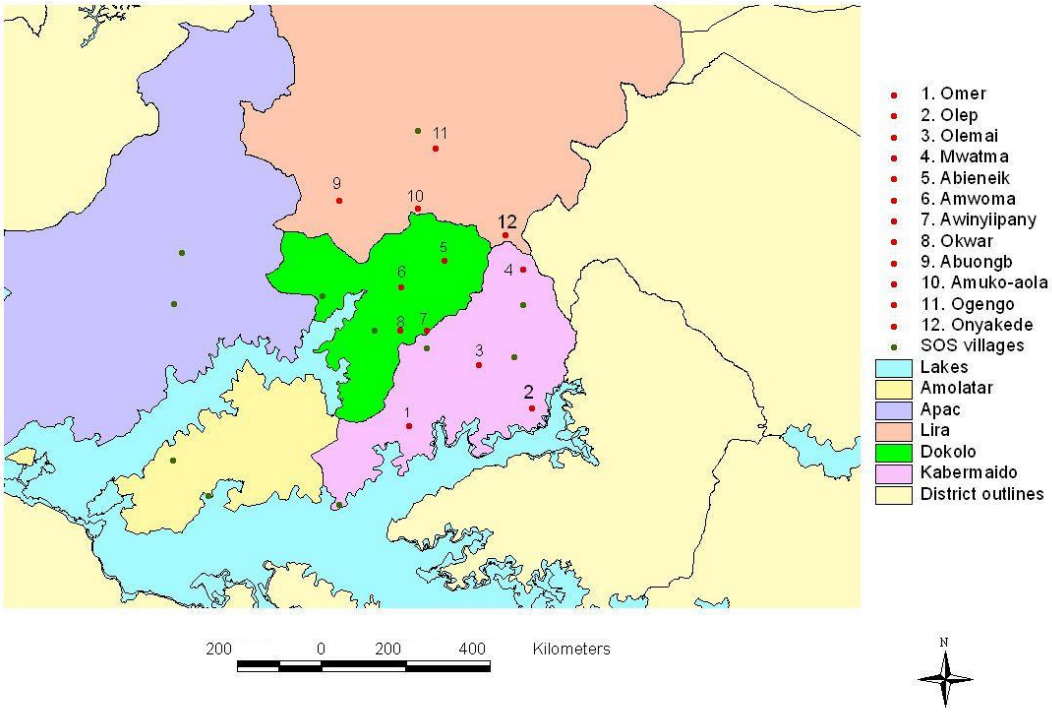

Figure 6.2: SOS project districts and the 23 sentinel villages with the selected 12 villages highlighted in red

6.3.3 Sampling

For sampling cattle, farmers were asked to present their animals to a central point of treatment. During both post-intervention samplings in the studied villages, farmers were asked whether the animal had been previously included in the SOS mass treatment programme. This information allowed for estimating the coverage of the mass treatment. The samples were collected by SOS team from Edinburgh University and Makarare University, Uganda. Table 6.1 shows the number of samples collected at each sampling site over the duration of the study.

Table 6.1: Number of samples at each sampling site selected in the current study over the three sampling times

Sampling sites				Baseline	Three months follow up	Nine months follow up
District	Sub county	Parish	Village			
Kabermaido	Kobulubulu	Okille	Omer	80	92	100
Kabermaido	Bululu	Ochelakur	Olep	80	92	76
Kabermaido	Anyara	Omid	Mwatma	80	100	46
Kabermaido	Otuboi	Lwala	Olemai	80	62	92
Dokolo	Kangai	Abwila	Okwar	80	82	100
Dokolo	Dokolo	Amwoma	Amwoma	100	59	100
Dokolo	Dokolo	Angweciba	Awinyi ipany	100	76	100
Dokolo	Batta	Abienek	Abienek	80	80	100
Lira	Amach	Adyaka	Abuong B	100	100	100
Lira	Abako	Owallo	Amuko aola	100	51	53
Lira	Aloi	Alal	Ogengo	80	71	59
Lira	Amugu	Ajonyi	Onyakede	80	49	100
Total			12	1040	914	1026

6.3.4 Geographical data

The locations of the villages were recorded using a hand-held Global Positioning System (GPS, Garmin, Olathe, KS, USA) at the site of sampling. The locations were entered into EXCEL spread sheet and saved as a DBF4 file. Geographical data of the districts and other shape files were obtained from Land and Surveys Department, Ministry of Land Housing and Urban Development of Uganda.

GPS data were collected in latitude/longitude format and were converted to the Biomass projection for processing of maps (Uganda Forest Department 1996). The parameters of the Biomass projection are (Map projection: Transverse Mercator, Spheroid: 1880, Central meridian: 33, Reference latitude: 0, Scale factor: 0.9996 and False easting: 500,000). Maps were produced using ArcView 3x Geographic Information System (ESRI Systems, Redlands, CA, USA).

6.3.5 Recording data

Data including the village name, age/sex/breed and body condition of the animal were recorded. Body condition scoring is a measure of the extent to which fat is stored or the muscle mass has declined. This can be measured by observing and examining the depth of the sub-lumbar fossa to assess the

extent of emaciation (Nicholson and Butterworth, 1986). During sampling, Zebu and Ankole cattle were scored for the body condition as follows: Fat (F), Medium (M) or Lean (L). The age of the animal was estimated based on teeth eruption; there are three categories as described in Table 6.2.

Table 6.2: Estimation of the animal age based on teeth eruption

Age category	Teeth eruption	Age
A (young)	Milk teeth erupted	Less than 18 months
B	One pair of permanent teeth	1.5- 3 years
C (adults)	More than one pair of permanent teeth	Over three years

6.3.6 Sample processing

Samples applied onto FTA®cards were processed by eluting DNA from 10 discs using Chelex®100 as previously described in Chapter IV (section, 4.5.2.1.2).

6.3.7 PCR

PCR reactions were used to amplify trypanosome DNA from 5 µl of DNA eluted from 10 discs using Chelex®100. The PCR reactions used were TBR-PCR for *T. brucei* s.l., TCS-PCR for *T. congolense* Savannah, TV-PCR for *T. vivax* and SRA-PCR for *T. b. rhodesiense*. The primer sets and reaction conditions were detailed in Chapter V (section, 5.2.3).

The primers specific for serum resistant associated gene (SRA) were used in a multiplex reaction with the PLC primers for the differentiation of *T. b. rhodesiense*. The sequence of the SRA primers are: 651: 5'- GAA GAG CCC GTC AAG AAG GTT TG-3' and 652: 5'- TTT TGA GCC TTC CAC AAG CTT GGG-3'. The reaction conditions are the same as for PLC-PCR (Chapter V, section 5.3.3). The amplified product using the SRA primers is 669 bp in size. There are some virulent surface glycoproteins (VSG) genes with some sequence identity to the SRA gene, but the SRA gene contains an internal deletion when compared with VSG genes (Campillo and Carrington, 2003). The aforementioned primers were designed to amplify the SRA gene across the deleted region, ensuring a clear size distinction between the SRA amplicon and any VSG amplicon (Picozzi *et al.*, 2008). Figure 6.3 shows a gel picture with PLC amplicon at 324 bp, SRA amplicon at 669 bp and VSG amplicon >1 kb.

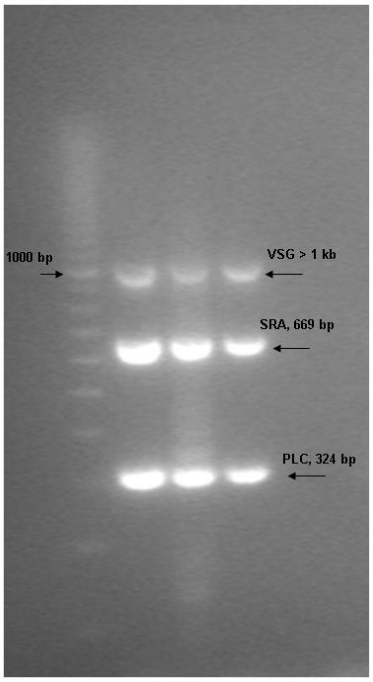

Figure 6.3: Example of multiplex reaction amplifying PLC and SRA genes in *T. b. rhodesiense* positive control (100 bp ladder, PLC amplicon is 324 bp, SRA amplicon is 669 bp and VSG amplicon is > 1 kb)

6.3.8 Statistical analysis

Trypanosome prevalence was calculated as the proportion of samples in which infection was detected by PCR at baseline, three months and nine months follow up, respectively. The 95% confidence interval was computed based on the exact binomial distribution and displayed as error bars in the respective graphs. The impact of the mass treatment campaign on different trypanosome species at the three sampling times across the study area and within districts was investigated using Odds ratios in R package and Chi-squared test (degree of freedom were noted as a subscript to the χ^2 –statistic). The prevalence, 95% CI and χ^2 test calculations were computed using Minitab version 15 (Minitab, Inc.). Differences were considered to be statistically significant at $p<0.05$.

To investigate the impact of the treatment campaign within the villages, Fisher's exact test was used and computed in R package. Differences were considered to be statistically significant at $p<0.05$.

6.4 Results

6.4.1 Sample description

A total of 2980 blood samples were collected from cattle in the three districts involved in the SOS mass-treatment programme at three time points over the whole study period. At baseline sampling, 1040 blood samples were collected, while 914 and 1026 were collected during both the three months and nine months post-intervention sampling, respectively. For each sample, information regarding age, sex, breed and condition score of the examined animal was collected and recorded. Information regarding age, sex and breed of some animals were not recorded, so those animals were excluded from the study. Table 6.3 summarises the proportion of the sampled animals in each age, sex, breed and condition category.

Table 6.3: Proportion of sampled animals at each age, sex, condition score and breed category at district level

District	Sampling time	Number sampled	Age			Sex		Condition			Breed		
			A	B	C	M	F	L	M	F	A	Z	Cross
Kabermaido	Baseline	320	7.5	29.1	63.1	47.5	52.2	4.7	86.9	8.4	20.0	65.0	15.0
	3 month	346	6.6	26.0	67.3	60.4	39.6	1.7	97.4	0.9	13.3	82.9	3.8
	9 month	314	7.0	16.9	75.8	58.0	42.0	3.5	95.9	0.6	17.2	76.8	5.4
	Total	980	7.0	24.1	68.7	55.4	44.5	3.3	93.5	3.3	16.7	75.1	8.0
Dokolo	Baseline	360	12.5	27.2	60.3	55.0	45.0	5.6	92.8	1.7	3.9	89.2	6.4
	3 month	297	7.4	26.3	66.3	62.0	38.0	5.4	92.3	2.4	7.7	87.5	4.7
	9 month	400	12.5	15.8	71.5	53.8	46.0	0.8	99.3	0.0	5.0	92.0	3.0
	Total	1057	11.1	22.6	66.2	56.5	43.4	3.7	95.1	1.2	5.4	89.8	4.6
Lira	Baseline	360	12.8	20.3	66.9	59.4	40.6	0.3	99.7	0.0	0.0	98.6	1.4
	3 month	271	10.7	22.9	66.4	56.8	43.2	4.1	93.7	2.2	1.8	96.3	1.8
	9 month	312	6.4	15.4	78.2	67.0	33.0	1.9	97.8	0.3	2.9	97.1	0.0
	Total	943	10.1	19.4	70.5	61.2	38.8	1.9	97.3	0.7	1.5	97.5	1.1
Total		2980	9.4	22.1	68.4	57.6	42.3	3.0	95.3	1.7	7.9	87.4	4.6

*** Age categories: A; less than 18 months, B; 1.5-3 years, C; over 3 years- Sex category: M; male, F; female- Condition score: L; lean, M; medium, F; fat- Breed category: A; Ankole, Z; Zebu, Cross; Zebu x Ankole or Freisian**

In the three districts, over the three sampling times, the majority of animals were of age over three years with a proportion of 68.4%, while the other age categories, less than 18 months and 1.5-3 years, were at a proportion of 9.4% and 22.1%, respectively. Most of the cattle sampled in the three districts over the sampling time were male animals (57.6%) compared to female animals (42.3%). During the three sampling times in the three districts, 95.3% of the sampled animals were of medium category condition score, whereas, only 1.7% and 3% were of fat and lean categories, respectively. The predominant breed of the examined animals were Zebu breed with 87.4% proportion, while, Ankole breed proportion was only 7.9%. The remaining proportion (4.6%) was of cross breeds, either Zebu x Ankole or Zebu x Freisian.

6.4.2 Baseline results

6.4.2.1 Overall prevalence of different trypanosome species at baseline in the sampled cattle

A total of 1040 cattle were sampled and examined for trypanosomes using PCR at baseline collection. Overall, 26.6% were found to be infected with different trypanosome species including *T. brucei* s.l., *T. vivax* and *T. congolense* Savannah with overall prevalences of 20.8%, 5.7% and 0.2%, respectively, mixed infection prevalence was 1.7%. The human infective *T. b. rhodesiense* species was identified in 1.3% of the examined animals.

Age of animal

Overall there was a significant difference between age groups on the prevalence of the overall parasitic events (χ_2^2=7, p=0.03). The highest prevalence was detected in the adult group (31.1%, 205/660; 95% CI: 27.5-34.7) followed by the 1.5-3 years age category (25%, 66/264; 95% CI: 19.9-30.7) and the young age group (20.9%, 24/115; 95% CI: 13.9-29.4). The difference between young and 1.5-3 years age groups was not significant (χ_1^2=0.8, p=0.4), while the difference between the adult and the young group was significant (χ_1^2=4.9, p=0.03). The difference between the adult and the 1.5-3 years age group (χ_1^2=3.3, p=0.07) was statistically insignificant. Looking at the prevalence of the individual species, there was a significant difference between age groups on the prevalence of *T. brucei* s.l. infection (χ_2^2=19.6, p<0.001) and *T. vivax* infection (χ_2^2=8.4, p=0.01). The prevalence of *T. brucei* s.l. infection was significantly higher (χ_1^2=5.6, p=0.02) in adults (24.5%, 162/660; 95% CI: 21.3-28) than in 1.5-3 years age group (17%, 45/264; 95% CI: 12.7-22.1). Also, the prevalence of *T. brucei* s.l. in adults was significantly higher (χ_1^2=15.9, p<0.001) compared to the prevalence in young calves (7.8%, 9/115; 95% CI: 3.6-14.3). The opposite was observed in *T. vivax* infections where the higher prevalence was in young calves (11.3%, 13/115; 95% CI: 6.2-18.6), followed by the 1.5-3 years age group (6.1%, 16/264; 95% CI: 3.5-9.7) then the adult group (4.5%, 30/660; 95% CI: 3.1-6.4). The difference in *T. vivax* prevalence between the young age group (less than 18 months) and the adult age group was significant (χ_1^2=8.5, p=0.003), while not significant between 1.5-3 years age group and the adult age group (χ_1^2=0.9, p=0.3) and between groups A/B (χ_1^2=3.1, p=0.08).

Gender of animals

No significant difference (χ_1^2=0.4, p=0.5) was observed between males and females in the prevalence of the overall parasitic events. Overall trypanosome species were found in 29.1% (164/564; 95% CI: 25.4-33) males and 27.4% (130/475; 95% CI: 23.4-31.6) females. In the case of the individual species, no statistical significant difference (χ_1^2=0.5, p=0.5) was found between the prevalence of *T. brucei* s.l. infections in male and female animals. The prevalence in males was 19.9% (112/564; 95% CI: 16.7-

23.4), while in females it was 21.7% (103/475; 95% CI: 18.1-25.7). However, a significant difference (χ_1^2=5.8, p=0.02) was observed between the prevalence of *T. vivax* with a higher prevalence in males 7.3% (41/564; 95% CI: 5.3-9.7) than in females 3.8% (18/475; 95% CI: 2.3-5.9).

Body condition score of animals

The overall prevalence of the parasitic events between the animal condition score groups was significant (χ_2^2=6.4, p=0.04) with the higher prevalence in lean animals (38.9%, 14/36; 95% CI: 23.1-56.5) followed by medium (28.5%, 277/971; 95% CI: 25.7-31.5) then fat animals (12.1%, 4/33; 95% CI: 3.4-28.2). There was no significant difference between the prevalence of the parasitic events in lean and medium condition scored animals (χ_1^2=1.8, p=0.2), however, the difference between the fat animals and the other two categories, lean (χ_1^2=6.4, p=0.01) and medium condition scored animals (χ_1^2=4.3, p=0.04) was significant.

Concerning the individual species, the prevalence of *T. brucei* infections was insignificant between the three categories (χ_2^2=5.4, p=0.07). The higher prevalence was observed in lean animals 27.8% (10/36; 95% CI: 14.2-45.2), which was significantly different from the prevalence in fat animals (χ_1^2=4.2, p=0.04). The prevalence in fat animals was 6.1% (2/33; 95% CI: 0.7-20.2) which was slightly different from the prevalence in medium scored animals (21%, 204/971; 95% CI: 18.5-23.7) approaching significance (χ_1^2=3.5, p=0.06). There was no statistical significant difference between the prevalence of *T. brucei* s.l. infections in lean and medium animals (χ_1^2=0.6, p=0.4). The condition score effect on the prevalence of *T. vivax* infections was insignificant when calculated using Fisher's exact test (p=0.6).

Animal breed

There was a significant difference in the prevalence of the overall parasitic events between the sampled animal breeds (χ_2^2=7.3, p=0.03). The highest prevalence was in Zebu cattle 30% (265/884; 95% CI: 27-33.1) followed by Cross breeds 21.1% (16/76; 95% CI: 12.5-31.9) then Ankole breeds 17.9% (14/78; 95% CI: 10.2-28.3). The only significant difference in the prevalence of any trypanosome species was observed only between Zebu and Ankole breeds (χ_1^2=5, p=0.03). By investigating the individual species, there was no significant effect of the animal breed on the prevalence of *T. brucei* s.l. infections (χ_2^2=4.1, p=0.1). The prevalence of *T. brucei* s.l. infections was higher in Zebu cattle 21.8% (193/884; 95% CI: 19.2-24.7) followed by Ankole 16.7% (13/78; 95% CI: 9.2-26.8) and Cross breeds 13.2% (10/76; 95% CI: 6.5-22.9).

Looking at the difference between animal breeds infected with *T. vivax* infections, there was a significant effect with p-value=0.04. Ankole breed was not infected with *T. vivax* infections, however, there was insignificant difference (p=0.8) between the prevalence of *T. vivax* infections in Zebu cattle 6.1% (54/884; 95% CI: 4.6-7.9) and cross breeds 6.6% (5/76; 95% CI: 2.2-14.7).

The prevalences of *T. congolense* Savannah, *T. b. rhodesiense* and mixed infections were not undertaken in the previous analysis due to low prevalences (0.2%, 1.3% and 1.7%, respectively).

6.4.2.2 Prevalence of different trypanosome species at district level

The results in Table 6.4 and Figure 6.4 summarise the prevalence of different trypanosome species at district level before the intervention programme.

Table 6.4: Baseline prevalence of different trypanosome species at district level [% (fraction; 95% CI)]

Trypanosome species	District			Overall
	Kabermaido	Dokolo	Lira	
T. brucei s.l.	16.9% (54/320; 12.9-21.4)	26.9% (97/360; 22.4-31.8)	18.1% (65/360; 14.2-22.4)	20.8% (216/1040; 18.3-23.4)
*T. b. rhodesiense**	0.9% (3/320; 0.2-2.7)	2.8% (10/360; 1.3-5.1)	0.3% (1/360; 0.007-1.5)	1.3% (14/1040; 0.7-2.2)
T. vivax	3.1% (10/320; 1.5-5.8)	4.2% (15/360; 2.4-6.8)	9.4% (34/360; 6.6-12.9)	5.7% (59/1040; 4.3-7.3)
T. congolense Savannah	0% (0/320; 0-0.9)	0.6% (2/360; 0.07-2)	0% (0/360; 0-0.8)	0.2% (2/1040; 0.02-0.7)
Mixed infection**	0.6% (2/320; 0.08-2.2)	1.7% (6/360; 0.6-3.6)	2.8% (10/360; 1.3-5.1)	1.7% (18/1040; 1-2.7)
Parasitic events	20% (64/320; 15.8-24.8)	31.7% (114/360; 26.9-36.7)	27.5% (99/360; 23-32.4)	26.6% (277/1040; 24-29.4)

*** The number of *T. b. rhodesiense* is included in the number of *T. brucei* s.l. infections **The number of mixed infection species is included in the relevant column**

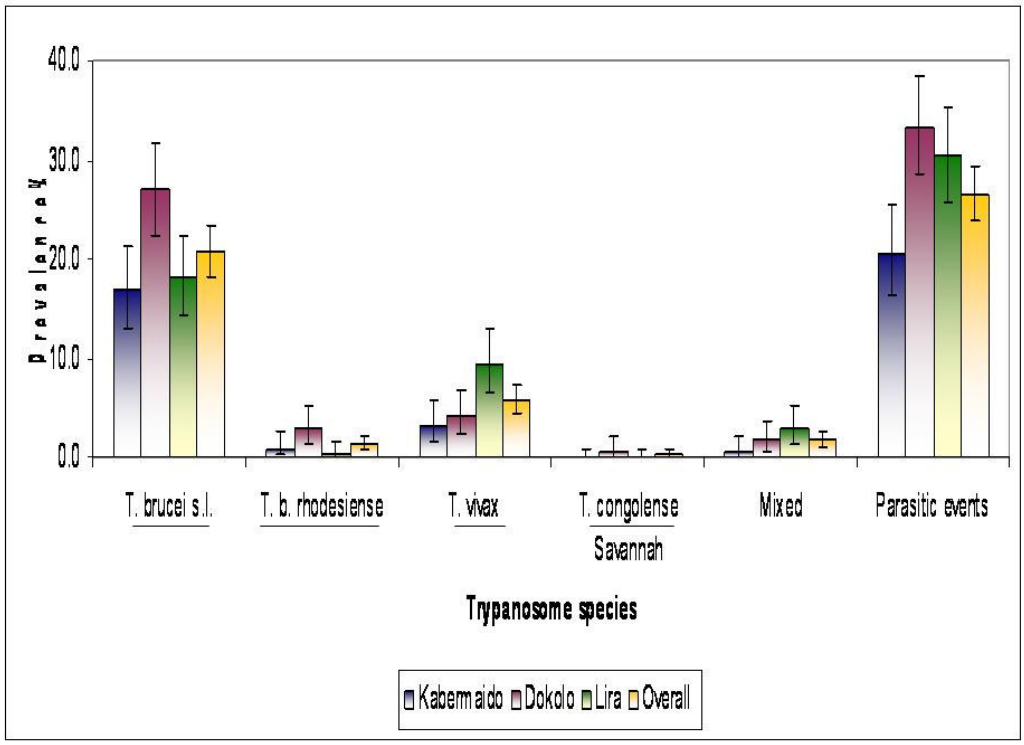

Figure 6.4: Baseline prevalence of trypanosome species at district level

The results in Table 6.4 and Figure 6.4 show the prevalence of trypanosome species identified using species-specific PCR at baseline sampling prior starting the intervention programme. A difference was observed in the prevalence of trypanosome species in the three districts (χ_2^2=14.4, p=0.001). The prevalence of the parasitic events in Kabermaido district (20%) was statistically lower than in Dokolo (χ_1^2=13.7, p<0.001, OR=0.5 (0.3-0.8)) and Lira (χ_1^2=8.3, p=0.004, OR=1.7 (1.1-2.6)). However, there was no significant difference between the prevalence of the parasitic events in Dokolo and Lira districts (χ_1^2 = 0.8, p=0.4, OR=0.9 (0.6-1.2)).

The results also show that *T. brucei* s.l. was the dominant species at baseline in the three districts compared to the other identified species. Overall, there was a significant difference between the prevalence of *T. brucei* s.l.*, T. b. rhodesiense, T. vivax, T. congolense* Savannah and mixed infections at baseline (χ_4^2=543.1, p<0.001). The prevalence of *T. brucei* s.l. was significantly different between the three districts (χ_2^2=12.9, p=0.002). The prevalence in Dokolo (26.9%) was significantly higher than the 18.1% prevalence observed in Lira (χ_1^2=8.2, p=0.004, OR=0.6 (0.4-0.9)) and16.9% prevalence in Kabermaido (χ_1^2=12.9, p=0.002, OR=0.6 (0.4-0.8)). No difference was observed

between Kabermaido and Lira in the prevalence of *T. brucei* s.l. infections (χ_1^2=0.16, p=0.6, OR=1.1 (0.6-1.8)).

The human infective trypanosome (*T. b. rhodesiense*) had an overall low prevalence (1.3%) at baseline. There was a significant difference between the three districts in the baseline prevalence of *T. b. rhodesiense* (p=0.01) with the highest prevalence in Dokolo district (2.8%) which was significantly higher than the 0.3% difference in Lira (p=0.01). However, the difference in *T. b. rhodesiense* prevalence was insignificant between Dokolo and Kabermaido (2.8% and 0.9%, respectively) with p-value of 0.09.

The overall baseline prevalence of *T. vivax* was 5.7% which was significantly different between the three districts (χ_2^2=14.9, p=0.001). Lira district had a significantly higher prevalence (9.4%) than Kabermaido district (χ_1^2=10.2, p=0.001, OR=3.2 (1.3-8)) which had 3.1% prevalence. Also, the difference in prevalence between Lira and Dokolo (9.4% and 4.2%, respectively) was statistically significant (χ_1^2=7.1, p=0.006, OR=2.4 (1.2-4.9)). However, no statistical significant difference was observed in the prevalence of *T. vivax* infections between Kabermaido and Dokolo (χ_1^2=0.51, p=0.5, OR=0.7 (0.3-1).

The other trypanosome species identified at a very low percentage using species-specific PCR was *T. congolense* Savannah. This species had an overall prevalence of 0.2% and was only identified in Dokolo district with 0.6% prevalence at baseline sampling.

Mixed infection was also identified in a low overall prevalence (1.7%) with no significant difference in the prevalence between the three districts (χ_2^2=14.9, p=0.1). The prevalence was higher in Lira district followed by Dokolo and Kabermaido (2.8%, 1.7% and 0.6%, respectively). The type of mixed infection identified in the three villages was *T. brucei* s.l./*T. vivax*; mixed infections did not include *T. b. rhodesiense*.

6.4.2.3 The prevalence of different trypanosome species at village level

The results in Figure 6.5 and 6.6 show the prevalence of *T. brucei* s.l. and *T. vivax* infections, respectively at village level.

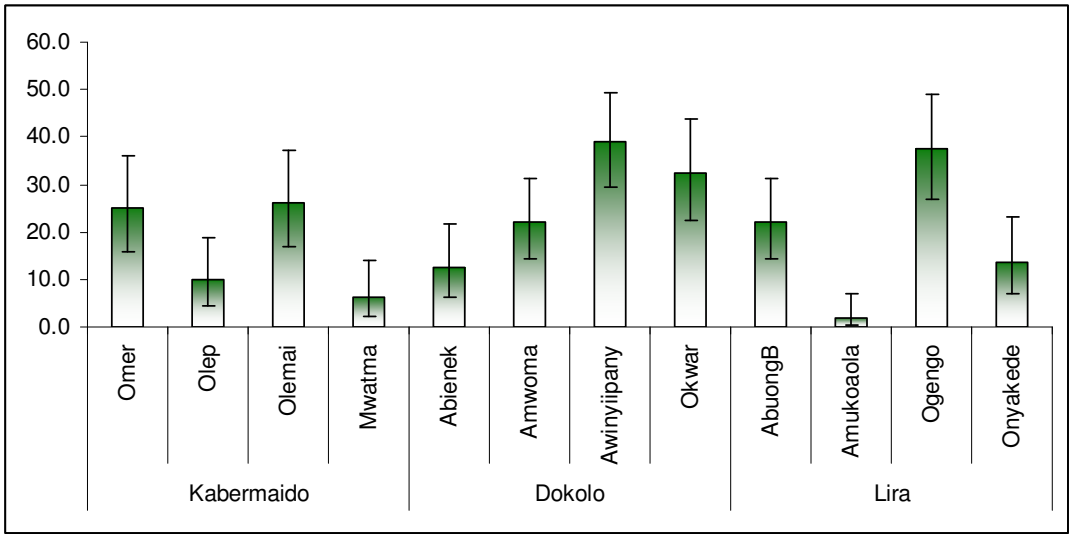

Figure 6.5: Prevalence of *T. brucei* s.l. at village level

The results in Figure 6.5 show the prevalence of *T. brucei* s.l. at the village level. The highest prevalence of *T. brucei* s.l. infection was in Awinyiipany (39%) and Okwar (32.5%) in Dokolo and Ogengo (37.5%) in Lira. However, a low prevalence of 6.3% and 2% were detected in Mwatma in Kabermaido and AmukoAola in Lira, respectively.

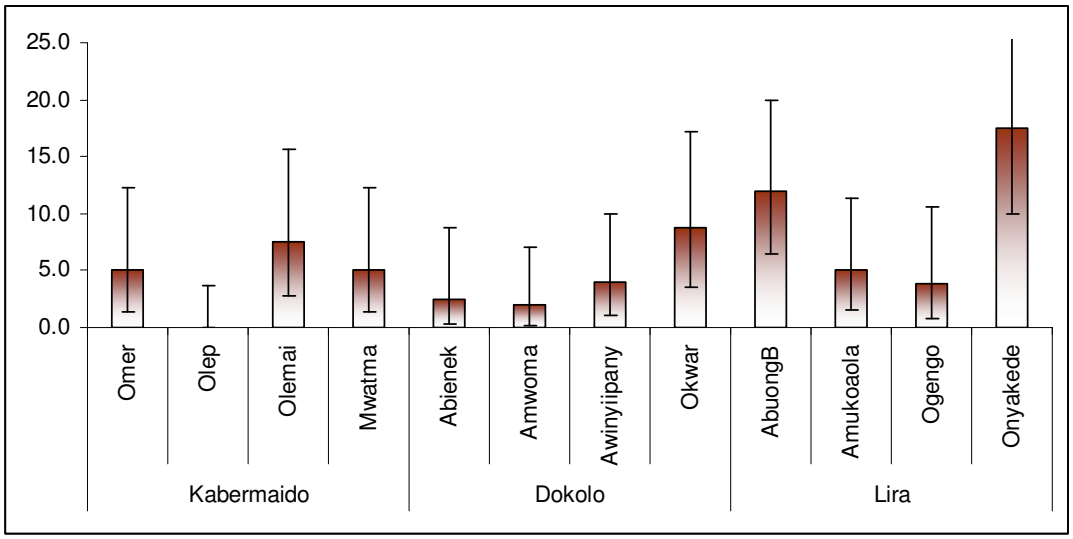

Figure 6.6: Prevalence of *T. vivax* at village level

The results in Figure 6.6 show the prevalence of *T. vivax* at the village level. Onyakede and AbuongB villages in Lira district had the highest prevalence of *T. vivax* infections (17.5% and 12%,

respectively). *T. vivax* was not identified in Olep in Kabermaido district and in the other villages prevalence ranged from 2%-12%.

T. congolense Savannah was only detected in Abienek village in Dokolo at baseline. Five villages had no mixed infections while other villages had a low prevalence of *T. brucei* s.l./*T. vivax* combination ranging from 1.3-5%.

6.4.3 Post intervention follow up results

6.4.3.1 Impact of intervention at the three sampling time points across the study area

The prevalence of different trypanosome species at baseline was compared with the prevalence in treated and non treated animals at three and nine months post-intervention to estimate the impact of the treatment programme. Table 6.5 and Figure 6.7 summarise the prevalence of different trypanosome species at baseline, three and nine months post-intervention.

Table 6.5: Prevalence of trypanosome species in animals across the study area [% (fraction; 95% CI)]

Sampling time	Description	*T. brucei* s.l.		*T. vivax*	*T. congolense* Savannah	Mixed infection**	Parasitic events
		T. brucei s.l.	*T. b. rhodesiense**				
Baseline	Pre-intervention	20.8% (216/1040; 18.3-23.4)	1.3% (14/1040; 0.7-2.2)	5.7% (59/1040; 4.3-7.3)	0.2% (2/1040; 0.02-0.7)	1.7% (18/1040; 1-2.7)	26.6% (277/1040; 24-29.4)
3 months	Treated	5.4% (40/737; 3.9-7.3)	0.1% (1/737; 0.003-0.8)	1.5% (11/737; 0.7-2.7)	0.1% (1/737; 0.003-0.8)	0 (0/737; 0-0.4)	7.1% (52/737; 5.3-9.1)
	Not treated	9.6% (8/83; 4.3-18.1)	0 (0/83; 0-3.5)	2.4% (2/83; 0.3-8.4)	0 (0/83; 0-3.5)	2.4% (2/83; 0.3-8.4)	12.04% (10/83; 5.9-21.04)
9 months	Treated	15.2% (96/630; 12.5-18.3)	1% (6/630; 0.4-2.1)	9.8% (62/630; 7.6-12.4)	0.6% (4/630; 0.2-1.7)	2.4% (15/630; 1.3-3.9)	25.7% (162/630; 22.3-29.3)
	Not treated	19.2% (47/245; 14.4-24.7)	0.4% (1/245; 0.01-2.3)	15.1% (37/245; 10.9-20.2)	0 (0/245; 0-1.2)	4.9% (12/245; 2.6-8.4)	34.3% (84/245; 28.4-40.6)

*** The number of *T. b. rhodesiense* is included in the number of *T. brucei* s.l. infections **The number of mixed infection species is included in the relevant column**

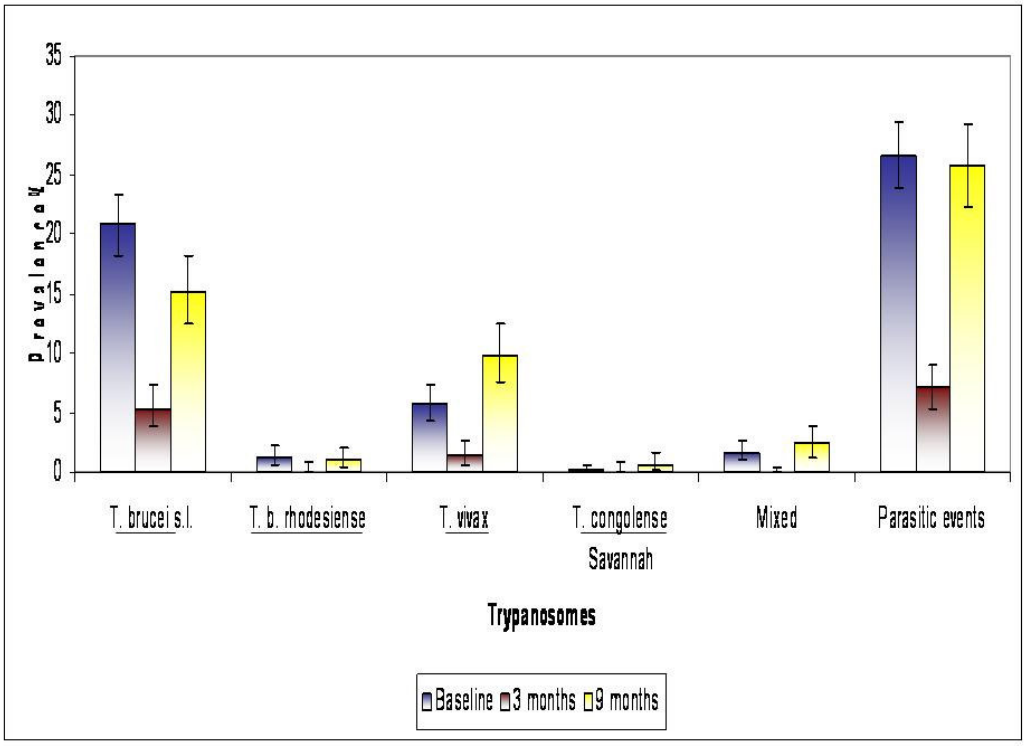

Figure 6.7: Prevalence of different trypanosome species in treated animals at baseline and post-intervention

The results in Table 6.6 and Figure 6.7 show that at baseline, the prevalence of the parasitic events was 26.6%, a significant reduction of the parasitic events prevalence was observed three months post-intervention reaching 7.1% (χ_1^2=124.6, p<0.001, OR=0.2 (0.1-0.3)). The prevalence of the parasitic events was significantly increased from 7.1% at three months post-intervention to 25.7% at nine months post-intervention (χ_1^2=107.8, p<0.001, OR=5.1 (3.6-7.2)). There was no significant difference between the baseline levels and those found nine months post intervention (χ_1^2=0.01, p=0.8, OR=0.9 (0.8-1.2)).

The prevalence of *T. brucei* s.l. infection significantly reduced from 20.8% at baseline to 5.4% three months post-intervention (χ_1^2=82.3, p<0.001, OR=0.2 (0.15-0.3)). The prevalence significantly increased to 15.2% after nine months (χ_1^2=36.5, p<0.001, OR=3.1 (2.1-4.7)), however, this increase was significantly lower than the baseline prevalence (χ_1^2=7.9, p=0.004, OR=0.7 (0.5-0.9)).

The prevalence of *T. vivax* infections at baseline (Table 6.6 and Figure 6.7) was 5.7%, which was found to be significantly reduced at three months post-intervention to 1.5% (χ_1^2=19.9, p<0.001, OR=0.3 (0.1-0.5)). Nine months post-intervention, there was a significant increase in the prevalence of *T. vivax* infections to 9.8% compared to three months post intervention (χ_1^2=46.8, p<0.001, OR=7.2

(3.6-14.4)). This increase was significantly higher compared to baseline prevalence (χ_1^2=10.1, p=0.001, OR=1.8 (1.2-2.7)).

The prevalence of *T. b. rhodesiense* significantly declined from 1.3% at baseline to 0.1% three months post-intervention (χ_1^2=7.6, p=0.006). Nine months post-intervention, the prevalence increased again to 1% (p=0.05), however, the prevalence at nine months post-intervention was insignificantly lower than that at baseline (p=0.6).

The same trend was observed on the impact of the treatment programme on *T. congolense* Savannah infections and mixed infection prevalence (Table 6.6 and Figure 6.7). Pre-intervention, the prevalence of *T. congolense* Savannah infections was 0.2% which insignificantly declined to 0.1% three months post-intervention (p=1). Nine months post-intervention, animals infected with *T. congolense* Savannah infections were detected with a prevalence of 0.6% which was insignificantly higher than the prevalence at baseline and three months post-intervention (p=0.2).

Mixed infection was identified at baseline with 1.7% prevalence which significantly was cleared three months post-intervention (χ_1^2=12.9, p<0.001). A significant re-appearance of mixed infection (2.4%) was detected nine months post-intervention (χ_1^2=17.7, p<0.001). The type of mixed infection detected at baseline was only *T. brucei* s.l./*T. vivax*, no *T. b. rhodeseiense* was involved in such mixed infection. However, at nine months post intervention 14 out of the detected 15 mixed infections were of *T. brucei* s.l./*T. vivax*, two of these combinations included *T. b. rhodeseinse.* Also, *T. congolense* Savannah was included in one case to have mixed infection with *T. brucei* s.l.

The prevalence of different trypanosome species at the three sampling time points in animals that had not received any intervention are listed in Table 6.5. These animals were used as controls to discuss and evaluate the impact of the treatment programme (Discussion section).

6.4.3.2 Impact of intervention at district level

The prevalence of different trypanosome species at district level during the treatment programme was compared to find out if there was a difference in the prevalence of trypanosome species in the three districts chosen for the study. The results of Olemai village were excluded from the follow up results due to low proportion of treated animals sampled three and nine months post-intervention (27.4% and 2.2%, respectively). Table 6.6 shows the prevalence of different trypanosomes in animals that received treatment in the three studied districts pre and post-intervention.

Table 6.6: Prevalence of different trypanosomes in the three districts pre and post-intervention [% (fraction; 95% CI)]

Sampling time	District	*T. brucei* s.l.		*T. vivax*	*T. congolense* Savannah	Mixed infection**	Parasitic events
		T. brucei s.l.	*T. b. rhodesiense**				
Kabermaido	Baseline	17% (54/320; 12.9-21.4)	0.9% (3/320; 0.2-2.7)	3.1% (10/320; 1.5-5.8)	0 (0/320; 0-0.9)	0.6% (2/320; 0.08-2.2)	20% (64/320; 15.8-24.8)
	3 months	9.4% (25/265; 6.2-13.6)	0.4% (1/265; 0.01-2.1)	0.8% (2/265; 0.09-2.7)	0 (0/265; 0-1.1)	0 (0/265; 0-1.1)	10.2% (27/265; 6.8-14.5)
	9 months	14.3% (26/182; 9.5-20.2)	0 (0/182; 0-1.6)	9.9% (18/182; 6-15.2)	1.6% (3/182; 0.3-4.7)	1.6% (3/182; 0.3-4.7)	25.8% (47/182; 19.6-32.8)
Dokolo	Baseline	26.9% (97/360; 22.4-31.8)	2.8% (10/360; 1.3-5.1)	4.2% (15/360; 2.4-6.8)	0.6% (2/360; 0.07-2)	1.7% (6/360; 0.6-3.6)	31.7% (114/360; 26.9-36.7)
	3 months	5.2% (13/252; 2.8-8.7)	0 (0/252; 0-1.2)	2% (5/252; 0.6-4.6)	0.4% (1/252; 0.01-2.2)	0 (0/252; 0-1.2)	7.5% (19/252; 4.6-11.5)
	9 months	16.1% (42/261; 11.9-21.1)	2.3% (6/261; 0.8-4.9)	7.7% (20/261; 4.7-11.6)	0.4% (1/261; 0.01-2.1)	3.1% (8/261; 1.3-6)	24.1% (63/261; 19.1-29.8)
Lira	Baseline	18.1% (65/360; 14.2-22.4)	0.3% (1/360; 0.007-1.5)	9.4% (34/360; 6.6-12.9)	0 (0/360; 0-0.8)	2.8% (10/360; 1.3-5.1)	27.5% (99/360; 23-32.4)
	3 months	0.9% (2/220; 0.1-3.2)	0 (0/220; 0-1.4)	1.8% (4/220; 0.5-4.6)	0 (0/220; 0-1.4)	0 (0/220; 0-1.4)	2.7% (6/220; 1-5.8)
	9 months	15% (28/187; 10.2-20.9)	0 (0/187; 0-1.6)	12.8% (24/187; 8.4-18.5)	0 (0/187; 0-1.6)	2.1% (4/187; 0.6-5.4)	27.8% (52/187; 21.5-34.8)

*** The number of *T. b. rhodesiense* is included in the number of *T. brucei* s.l. infections **The number of mixed infection species is included in the relevant column**

6.4.3.2.1 Prevalence of the overall parasitic events

Table 6.6 and Figure 6.8 show the prevalence of the parasitic events detected using species-specific PCR in the three districts all over the time of the study pre and post-intervention.

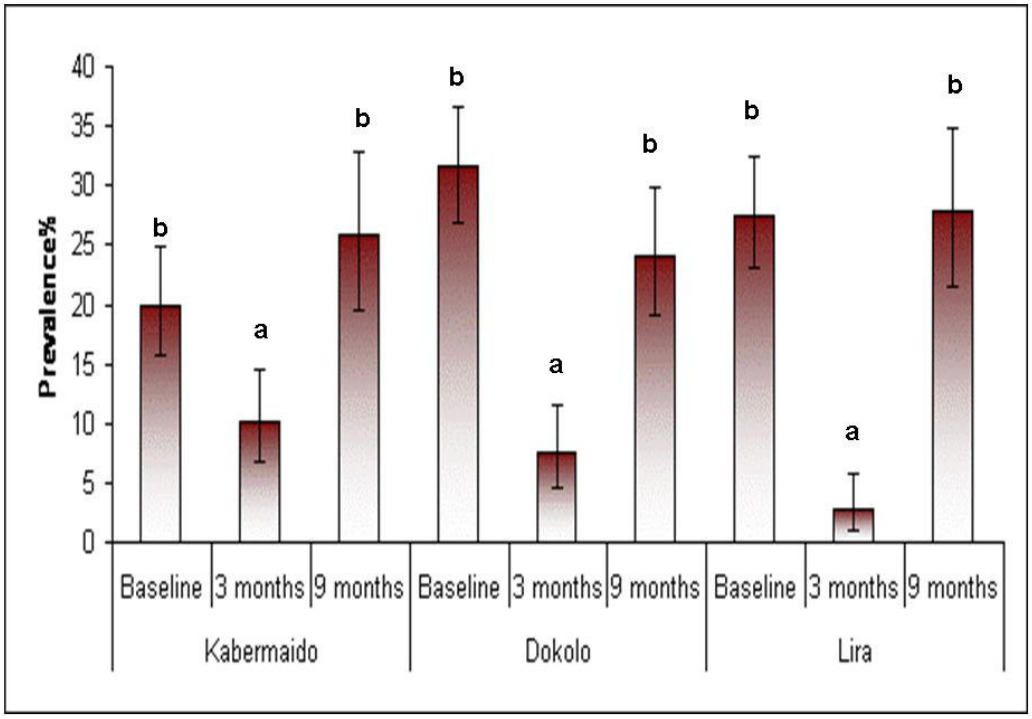

Figure 6.8: Prevalence of the parasitic events at the three studied districts (a= significant difference from baseline, b= significant difference from 3 months)

The results in Table 6.6 and Figure 6.8 show that the prevalence of the parasitic events in the three districts was reduced three months post-intervention in each district. In Kabermaido district, the prevalence of the parasitic events declined significantly from 20% at baseline to 10.2% three months post-intervention (χ_1^2=11.1, p=0.001, OR=0.4 (0.2-0.8)). The prevalence significantly increased again to 25.8% nine months post-intervention (χ_1^2=21.4, p<0.001, OR=3.2 (1.6-6.3)). However, the prevalence at nine months was not significantly higher than the baseline prevalence (χ_1^2=2.7, p=0.1, OR=1.4 (0.8-2.3)).

The same trend was noticed in Dokolo district where the prevalence of the parasitic events significantly reduced from 31.7% at baseline to 7.5% three months post-intervention (χ_1^2=54.7, p<0.001, OR=0.2 (0.01-0.3)). Nine months post-intervention the prevalence increased significantly to 24.1% compared to three months post-intervention (χ_1^2=32.9, p<0.001, OR=4.6 (2.3-9.01). The prevalence at nine months was not significantly higher than the pre-intervention prevalence (χ_1^2=2.4, p=0.1, OR=0.7 (0.5-1.1)).

Finally, in Lira district, the prevalence of the parasitic events was reduced significantly from 27.5% at baseline to 2.7% three months post-intervention (χ_1^2=63.5, p<0.001, OR=0.1 (0.02-0.2)). The

prevalence significantly increased again nine months post-intervention to 27.8% (χ_1^2=55.9, p<0.001, OR=15.2 (5.1-45.1)) which was not significantly lower than the prevalence at baseline (χ_1^2=0.01, p=0.9, OR=1 (0.6-1.5)).

At three months post-intervention, there was a significant difference in the prevalence of the parasitic events between the three districts (χ_2^2=10.3, p=0.006). The 2.7% prevalence in Lira district was significantly lower than the 7.5% prevalence in Dokolo (χ_1^2=4.5, p0.03, OR=0.3 (0.1-1.01)) and the 10.2% prevalence in Kabermaido (χ_1^2=9.4, p=0.002, OR=0.2 (0.1-0.8)). However, there was insignificant difference between Kabermaido and Dokolo in the prevalence of the parasitic events (χ_1^2=0.8, p=0.4, OR=1.4 (0.7-2.8)).

Nine months post-intervention, there was no statistical significant difference between the three districts in the prevalence of the parasitic events (χ_2^2=10.3, p=0.006). The detected prevalence of trypanosome species in Kabermaido, Dokolo and Lira was 25.8%, 24.1% and 27.8%, respectively.

6.4.3.2.2 *T. brucei* s.l. prevalence

Figure 6.9 shows the prevalence of infections with *T. brucei* s.l. at the three studied districts over the three sampling periods.

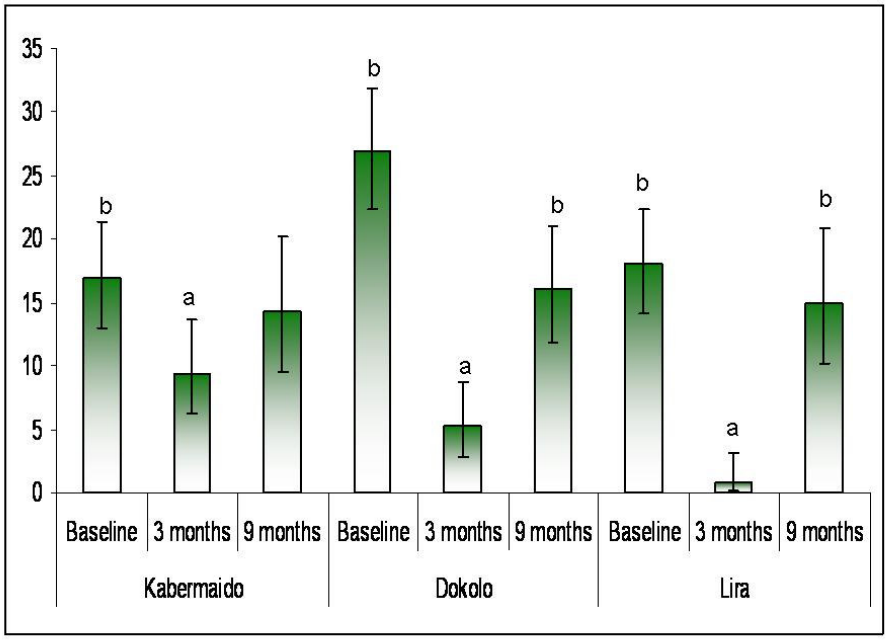

Figure 6.9: Prevalence of *T. brucei* s.l. at the three studied districts pre and post-intervention (a= significant difference from baseline, b= significant difference from 3 months)

The results in Table 6.6 and Figure 6.9 show that the prevalence of *T. brucei* s.l. in Kabermaido was significantly reduced from 17% at baseline to 9.4% three months post-intervention (χ_1^2=6.2, p=0.01, OR=0.5 (0.3-0.9)). Although the prevalence increased again to 14.3% nine months post-intervention, this was insignificant compared to three months (χ_1^2=2.1, p=0.2, OR=1.5 (0.7-3.4). The prevalence at nine months follow up was insignificantly lower than the baseline prevalence (χ_1^2=0.4, p=0.5, OR=0.8 (0.4-1.5)). However, in Dokolo, the prevalence of *T. brucei* s.l. was significantly reduced from 26.9% at baseline to 5.2% three months post-intervention (χ_1^2=46.3, p<0.001, OR=0.14 (0.1-0.3)). The prevalence significantly increased again to 16.1% nine months post-intervention (χ_1^2=14.9, p<0.001, OR=3.5 (1.6-7.9)) when compared with the prevalence at three months post-intervention. The prevalence after the treatment by nine months was significantly lower than the prevalence at baseline (χ_1^2=9.6, p=0.002, OR=0.5 (0.3-0.8)). Lira district, the prevalence of *T. brucei* s.l. was significantly reduced from 18.1% at baseline to 0.9% nine months after treatment (χ_1^2=37.6, p<0.001, OR=0.04 (0.01-0.2)). The prevalence significantly increased again to 15% nine months after the treatment programme when compared with the prevalence at three months (χ_1^2=27.3, p<0.001, OR=19.2 (3.1-117.2)). However, the prevalence at nine months post intervention was insignificantly lower than the prevalence at baseline (χ_1^2=0.6, p=0.4, OR=0.8 (0.5-1.4)).

Three months post-intervention, there was a significant difference in the prevalence of *T. brucei* s.l. infections between the three districts (χ_2^2=17.1, p<0.001). The prevalence in Lira (0.9%) was significantly lower than the 9.4% prevalence in Kabermaido (χ_1^2=15.03, p=0.001, OR=0.1 (0.01-0.5)). The prevalence of *T. brucei* s.l. in Lira was lower than 5.2% prevalence in Dokolo, the difference was significant (χ_1^2=5.6, p=0.02, OR=0.2 (0.03-1)). However, there was no significant difference in *T. brucei* s.l. prevalence between Kabermaido and Dokolo districts (χ_1^2=2.9, p=0.07, OR=1.9 (0.9-4.3)).

The prevalence of *T. brucei* s.l. in Kabermaido, Dokolo and Lira districts nine months post-intervention was 14.3%, 16.1% and 15%, respectively. No statistical significant difference was observed in the prevalence of *T. brucei* s.l. at nine months post-intervention between the three districts (χ_2^2=0.3, p=0.9).

6.4.3.2.3 *T. b. rhodesiense* prevalence

As shown in Table 6.6, the prevalence of the human infective *T. b. rhodesiense* in Kabermaido district insignificantly decreased from 0.9% at baseline to 0.4% three months post-intervention (p=0.6). At nine months follow-up, no *T. b. rhodesiense* was detected in Kabermaido. However, in Dokolo district, the drop in *T. b. rhodesiense* prevalence was significant from 2.8% at baseline to a complete absence at three months follow up (p=0.007). The prevalence significantly increased again to 2.3% nine months post-intervention (p=0.03) which is insignificantly higher than the baseline prevalence (p=0.8). The animals sampled in Lira district post-intervention were free from the human infective parasite *T. b. rhodesiense*. The fall of *T. b. rhodesiense* following the intervention from 0.3% baseline prevalence was not significant (p=1).

6.4.3.2.4 *T. vivax* prevalence

Figure 6.10 shows the prevalence of infections with *T. vivax* at the three districts studied over the three sampling periods.

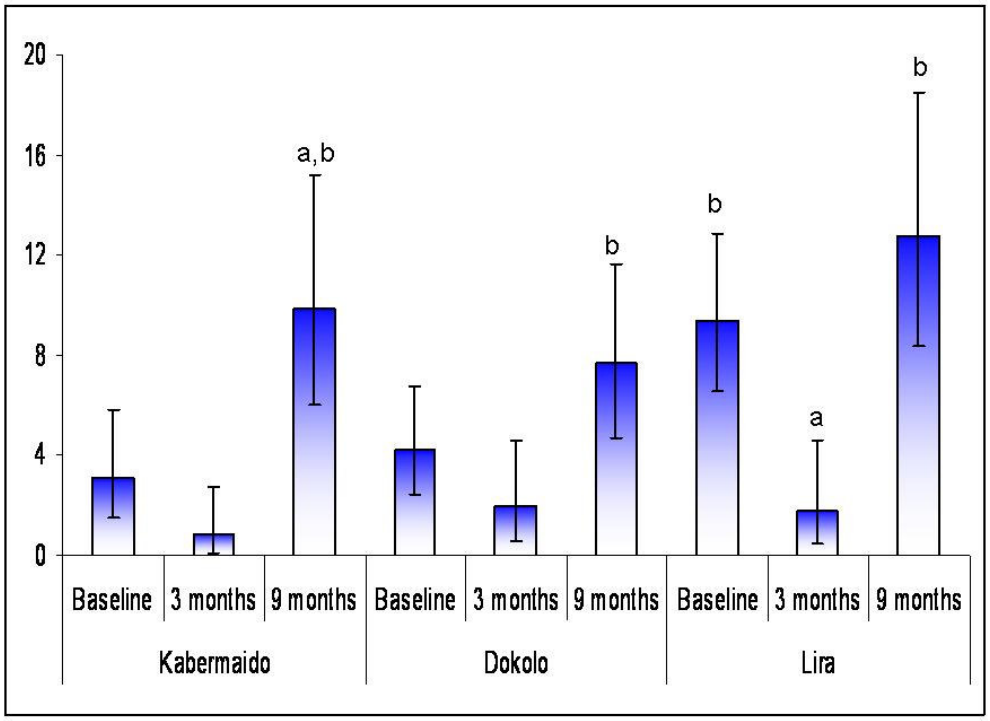

Figure 6.10: Prevalence of *T. vivax* in the three districts over the study period (a= significant difference from baseline, b= significant difference from 3 months)

The prevalence of *T. vivax* infections in the three districts pre and post-intervention is shown in Table 6.6 and Figure 6.10. The prevalence of *T. vivax* infections in Kabermaido was insignificantly decreased from 3.1% pre-intervention to 0.8% three months post-intervention (χ_1^2=3, p=0.1, OR=0.2 (0.04-1.4)). Nine months post-intervention, the prevalence significantly increased again to 9.9% when compared to the three months prevalence (χ_1^2=19, p<0.001, OR=14.6 (2.1-101)). The nine months prevalence of *T. vivax* infections was also significantly higher than the baseline prevalence (χ_1^2=8.8, p=0.002, OR=3.4 (1.4-8.8)). In Dokolo, the 4.2% baseline prevalence insignificantly decreased at three months post-intervention reaching 2% (χ_1^2=1.6, p=0.14, OR=0.5 (0.14-1.5)). The prevalence rose significantly nine months post-intervention reaching 7.7% (χ_1^2=7.7, p=0.005, OR=4.1 (1.2-14.2)). The prevalence at nine months post-intervention was insignificantly higher than the prevalence at baseline (χ_1^2=2.9, p=0.1, OR=1.9 (0.9-4.2)).

Finally, the prevalence of *T. vivax* infections in Lira district was significantly reduced from 9.4% at baseline to 1.8% three months post-intervention (χ_1^2=11.8, p=0.001, OR=0.2 (0.1-0.6)). Significantly, the prevalence increased again nine months post-intervention to 12.8% when compared to the three months post-intervention prevalence (χ_1^2=17.5, p<0.001, OR=8 (2.1-30.5)). However, the prevalence nine months post-intervention was insignificantly higher than the prevalence at baseline (χ_1^2=1.2, p=0.2, OR=1.4 (0.7-2.7)).

Three months post-intervention there was insignificant difference in the prevalence of *T. vivax* infections between the three districts (p=0.5). The prevalence of *T. vivax* infections at three months follow up was 0.8%, 2% and 1.8% in Kabermaido, Dokolo and Lira, respectively. Moreover, there was no statistical significant difference in the prevalence of *T. vivax* infections between the three districts at nine months follow up (χ_1^2=3.3, p=0.2). The respective prevalence of *T. vivax* infections at nine months follow up was 9.9%, 7.7% and 12.8% in Kabermaido, Dokolo and Lira.

6.4.3.2.5 *T. congolense* Savannah prevalence

Dokolo district was the only district where *T. congolense* Savannah infections were identified pre and post-intervention. The prevalence at baseline was 0.6% which insignificantly decreased to 0.4% three months post-intervention (p=1). The prevalence at nine months follow up was not changed from 0.4%. In Kabermaido district, *T. congolense* Savannah was only identified at nine months follow-up (1.6%), while, none were detected in Lira district over the study period (Table 6.6).

6.4.3.2.6 Mixed infection

The prevalence of mixed infection had the same trend as other species over the studied area during the treatment campaign. In Kabermaido, the prevalence of mixed infection was 0.6% at baseline, while none was identified three months post-intervention. The prevalence rose again at nine months follow up reaching 1.6% which was insignificantly higher than the baseline prevalence (p=0.4). The type of mixed infection in Kabermaido at baseline and three months post-intervention was *T. b. brucei/T. vivax* with no *T. b. rhodesiense* involved.

The prevalence of mixed infection in Dokolo at baseline was 1.7% with none being detected at three months follow-up, however, the prevalence increased again nine months post-intervention to 3.1%. The type of mixed infection at baseline was *T. b. brucei/T. vivax*. Eight animals were diagnosed to harbour mixed infection nine months post-intervention. One animal had *T. b. brucei/T. congolense* Savannah combination; two animals had *T. b. rhodesiense/T. vivax* combination. While the remaining six animals had *T. b. brucei/T. vivax* mixed infection. In Lira district, 2.8% and 2.1% prevalence of *T. brucei/T. vivax* mixed infection were identified at baseline and nine months post-intervention, respectively (Table 6.6).

6.4.3.3 Impact of intervention at village level

The prevalence of different trypanosome species at village level during the treatment programme was compared to find out if there was a difference in the prevalence of trypanosomes in the 11 villages chosen for the study. Olemai village has been excluded from the analysis due to the low number of treated animals at three and nine months post-intervention.

6.4.3.3.1 Prevalence of the overall parasitic events

Figure 6.11 show the prevalence of the overall events at village level.

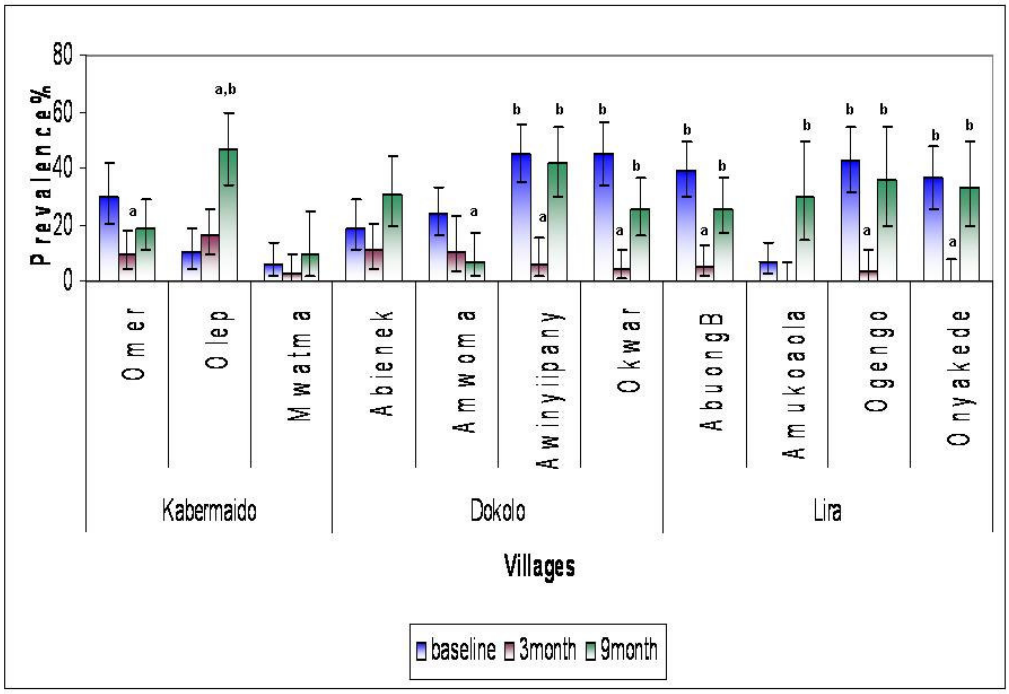

Figure 6.11: Prevalence of the overall parasitic events at village level at the three time points (a= significant difference from baseline, b= significant difference from 3 months)

6.4.3.3.2 *T. brucei* s.l. infection

Figure 6.12 show the prevalence of *T. brucei* s.l. at village level.

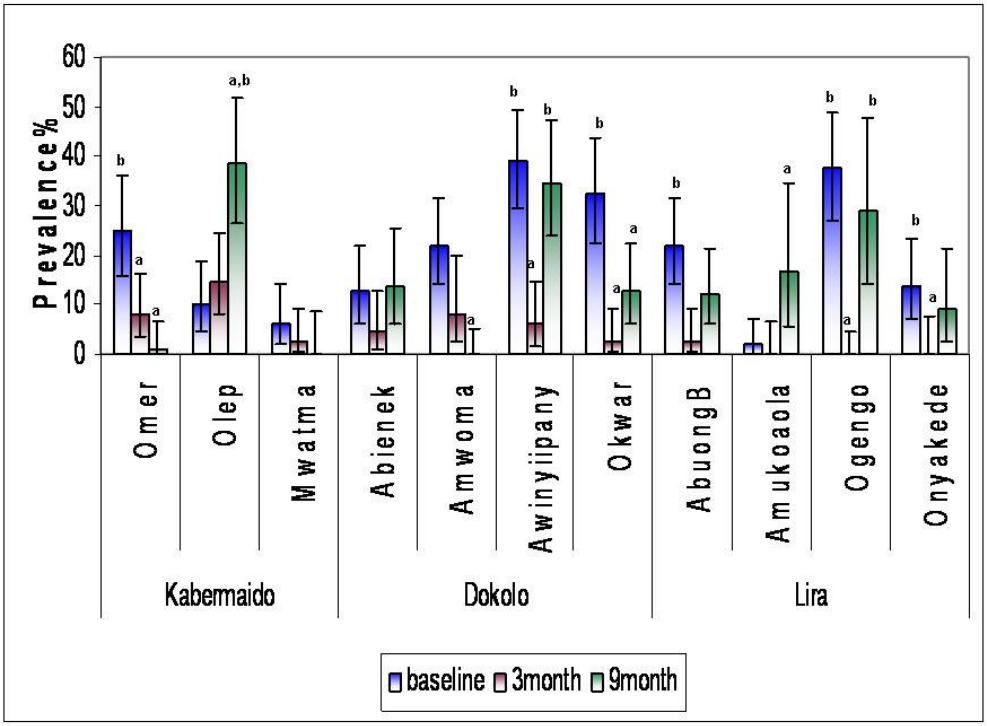

Figure 6.12: Prevalence of *T. brucei* s.l. at village level at the three time points (a= significant difference from baseline, b= significant difference from 3 months)

6.4.3.3.3 *T. vivax* infection

Figure 6.13 show the prevalence of *T. vivax* at village level.

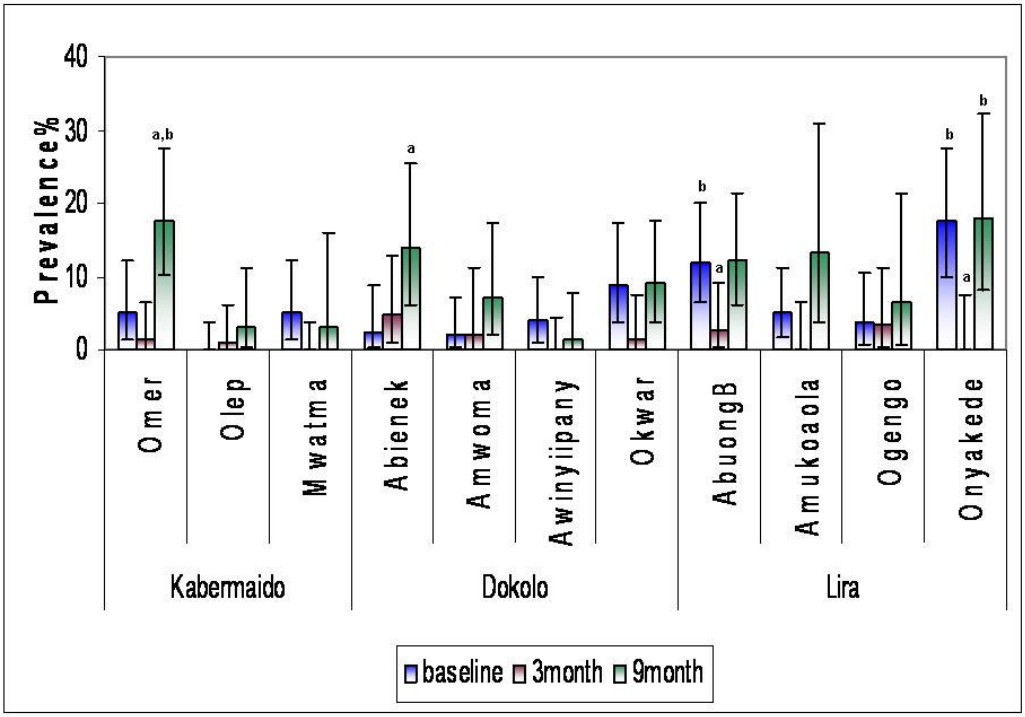

Figure 6.13: Prevalence of *T. vivax* at village level at the three time points (a= significant difference from baseline, b= significant difference from 3 months)

6.4.3.3.4 *T. congolense* Savannah and mixed infection

Infections with *T. congolense* Savannah were identified at the three sampling time points; Figure 5.14 shows the location where *T. congolense* Savannah was isolated.

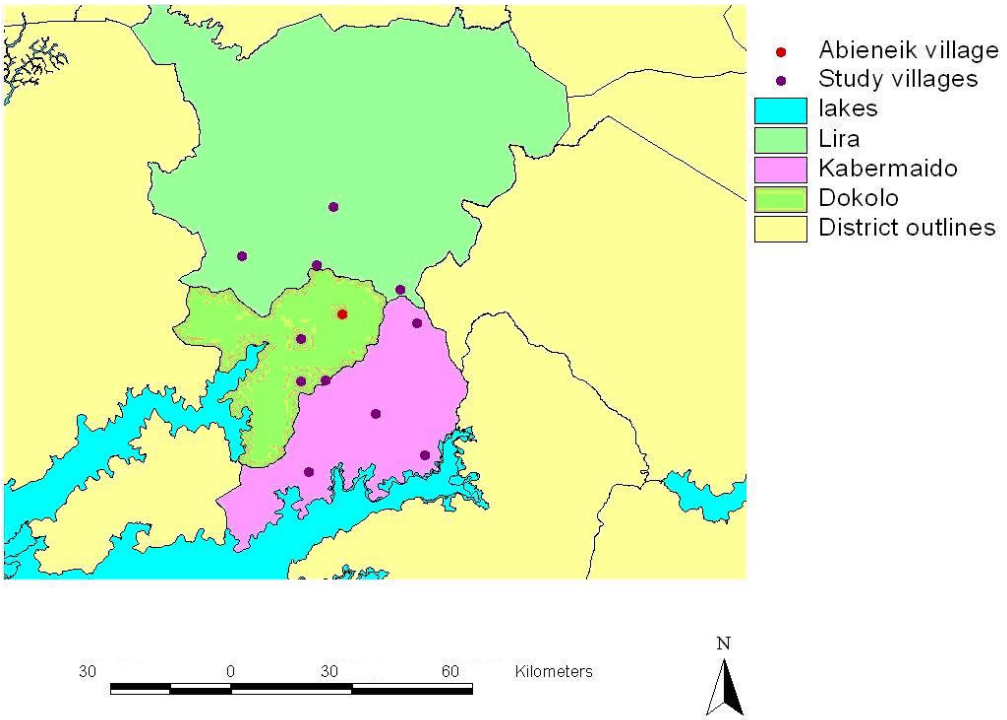

Figure 6.14: Map showing the village highlighted in red with *T. congolense* Savannah infected cattle at baseline and three months post-intervention

Figure 5.14 shows that animals infected with *T. congolense* Savannah infections were identified in Abieneik village only at baseline and three months post-intervention. The baseline prevalence was 2.5% (2/80; 95% CI: 0.3-8.7) which insignificantly (p=1) decreased to 1.5% (1/65; 95% CI: 0.04-8.3) three months post-intervention.

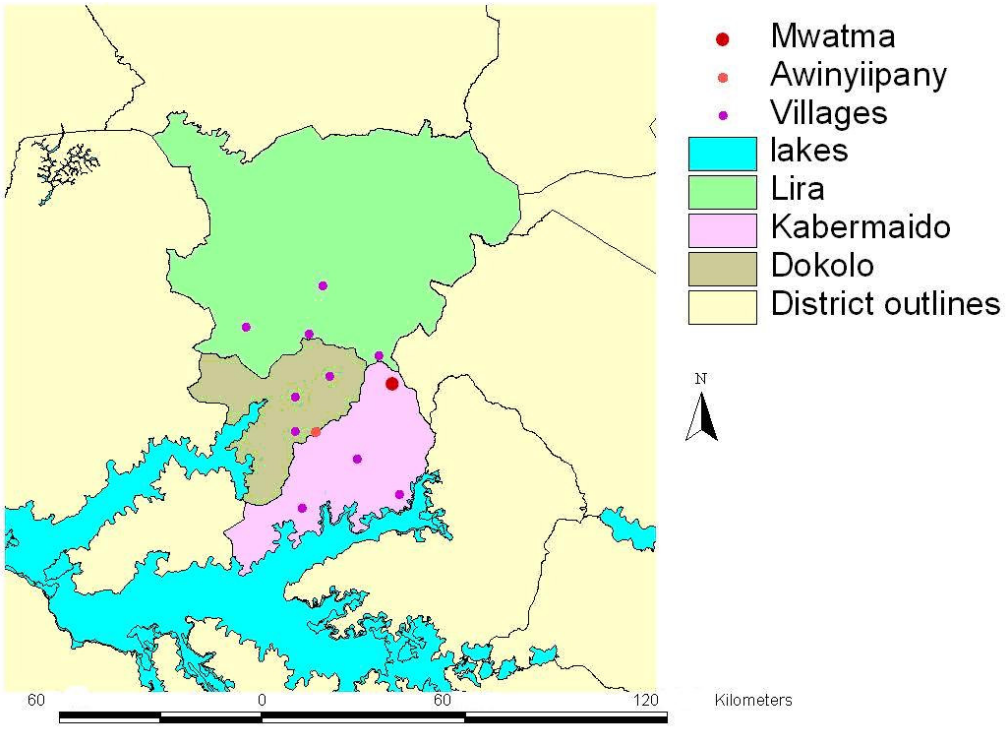

Figure 6.15: Map showing the two villages highlighted in red (the size and deepness of the colour is related to the prevalence) with *T. congolense* Savannah infected animals nine months after treatment

Figure 6.15 shows the villages with animals infected with *T. congolense* Savannah. Interestingly, Abieneik village was completely cleared from *T. congolense* Savannah infections after being infected at baseline and three months post-intervention. However, new cases with *T. congolense* Savannah infection were identified in new villages nine months post-intervention. The two villages with the new *T. congolense* Savannah cases were Mwatma and Awinyiipany with prevalence of 9.1% (3/33; 95% CI: 1.9-24.3) and 1.4% (1/69; 95% CI: 0.04-7.8), respectively.

Mixed infections were identified from the studied villages at baseline and nine months post-intervention. However, three months post-intervention mixed infections were cleared from the animals. Figures 6.16, 6.17 show the villages with mixed infections at baseline and nine months post-intervention.

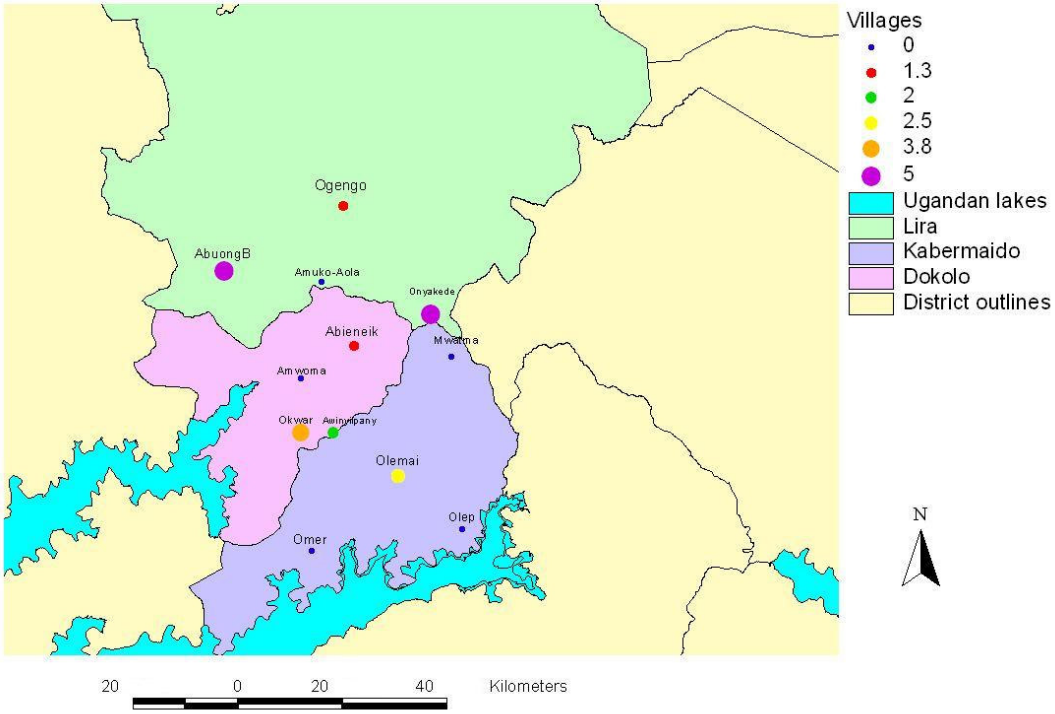

Figure 6.16: Map showing the baseline prevalence of mixed infection with the size of village symbols graduated according to the prevalence

Figure 6.16 shows that animals with mixed infection were identified at baseline in seven villages. The prevalence of mixed infection was 5% in AbuongB and Onyakede villages, while it was 3.8% in Okwar. A prevalence of 1.3% was detected in Abieneik and Ogengo villages; however, 2% and 2.5% prevalence were detected in Awinyiipany and Olemail villages, respectively. The type of mixed infection identified in all villages was *T. b. brucei/T. vivax* combination.

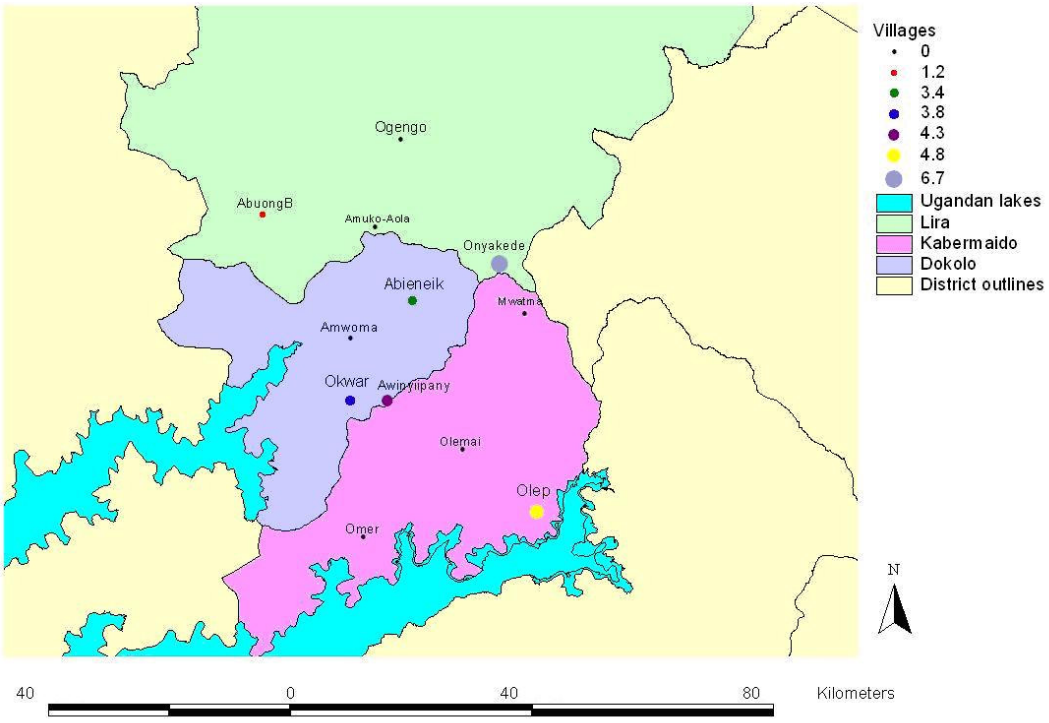

Figure 6.17: Map showing the prevalence of mixed infection at nine months post-intervention with the size graduated according to the prevalence

Figure 5.17 shows that nine months post-intervention, mixed infection with trypanosome species was detected again after being cleared from the animals three months following the intervention. Olemai village was excluded due to the low treatment coverage. Nine months post-intervention, the prevalence of mixed infection in AbuongB village raised again reaching 1.2% which was insignificantly lower than the baseline prevalence (p=0.2). In Onyakede, Abieneik and Awinyiipany villages, mixed infection prevalence was not significantly higher than the baseline prevalence (p=1). However, the prevalence in Okwar village increased again to the same level as at baseline. In Olep village, mixed infection was detected only at nine months post-intervention with 4.8% prevalence. Only Awinyiipany village had one animal harbouring *T. b. brucei/T. congolense* Savannah combination. Moreover, Okwar village had two animals infected with *T. b. rhodesiense/T. vivax* combination. The type of mixed infection in the other villages was *T. b. brucei/T. vivax*.

6.4.3.4 Impact of the drugs used on the prevalence of infections with trypanosome species

The impact of ISM and DI on the prevalence of infections with trypanosome species was studied by comparing the prevalence of trypanosomes at the three sampling times. Table 6.7 summarises the prevalence of different trypanosome infections during the campaign using the two drugs.

Table 6.7: Impact of ISM and DI on different trypanosome species

Drug	Sampling time	*T. brucei* s.l.		*T. vivax*	*T. congolense* Savannah	Mixed infection**	Parasitic events
		T. brucei s.l.	*T. b. rhodesiense*				
ISM	Baseline	22.2% (151/680; 19.1-25.5)	1.9% (13/680; 1-3.2)	3.7% (25/680; 2.4-5.4)	0.3% (2/680; 0.04-1.1)	1.2% (8/680; 0.5-2.3)	26.2% (178/680; 22.9-29.7)
	3 months	7.4% (38/517; 5.3-9.9)	0.2% (1/517; 0.005-1.1)	1.4% (7/517; 0.5-2.8)	0.2% (1/517; 0.005-1.1)	0% (0/517; 0-0.6)	8.9% (46/517; 6.6-11.7)
	9 months	15.3% (68/443; 12.1-19.1)	1.4% (6/443; 0.5-2.9)	8.6% (38/443; 6.1-11.6)	0.9% (4/443; 0.2-2.3)	2.5% (11/443; 1.2-4.4)	24.8% (110/443; 20.9-29.1)
DI	Baseline	18.1% (65/360; 14.2-22.4)	0.3% (1/360; 0.007-1.5)	9.4% (34/360; 6.6-12.9)	0 (0/360; 0-0.8)	2.8% (10/360; 1.3-5.1)	27.5% (99/360; 23-32.4)
	3 months	0.9% (2/220; 0.1-3.2)	0 (0/220; 0-1.4)	1.8% (4/220; 0.5-4.6)	0 (0/220; 0-1.4)	0 (0/220; 0-1.4)	2.7% (6/220; 1-5.8)
	9 months	15% (28/187; 10.2-20.9)	0 (0/187; 0-1.6)	12.8% (24/187; 8.4-18.5)	0 (0/187; 0-1.6)	2.1% (4/187; 0.6-5.4)	27.8% (52/187; 21.5-34.8)

*** The number of *T. b. rhodesiense* is included in the number of *T. brucei* s.l. infections **The number of mixed infection species is included in the relevant column**

The results in Table 6.7 show that at areas treated with ISM, there was a significant reduction of the overall parasitic events prevalence from 26.2% at baseline to 8.9% three months post-intervention (χ_1^2=64.02, p<0.001). Moreover, the significant reduction of *T. brucei* s.l. (χ_1^2=48.7, p<0.001), *T. b. rhodesiense* (χ_1^2=7.5, p=0.006) and *T. vivax* (χ_1^2=6.1, p=0.01) prevalence three months post-intervention was reported in areas treated with ISM. Also, there was a significant reduction in the prevalence of mixed infection from 1.2% at baseline to complete absence three months later (p=0.001). There was no statistically significant effect of ISM on the prevalence of *T. congolense* Savannah infections.

Areas treated with DI showed a significant reduction of the parasitic events prevalence from 27.5% at baseline to 2.7% three months post-intervention (χ_1^2=65.2, p<0.001). The significant impact of DI on reducing the prevalence of *T. brucei* s.l. (χ_1^2=39.3, p<0.001) and *T. vivax* (χ_1^2=13, p<0.001) infections was clearly obvious three months post-intervention (Table 6.7). Moreover, mixed infections were significantly reduced from 2.8% prevalence at baseline to complete absence three months later in areas treated with DI (p=0.02). In contrast, ISM and DI effect on the human infective trypanosome species *T. b. rhodesiense* was statistically insignificant (p=1). This might be due to the low level of *T. b. rhodesiense* detected at baseline (1.9%).

6.5 Discussion

6.5.1 Baseline results

The aim of the study presented in this chapter was to apply the sample preparation and molecular approaches evaluated in Chapters IV and V in field to monitor the effect of mass treatment programme on the prevalence of trypanosome species.

6.5.1.1 Overall prevalence of different trypanosome species at baseline in the sampled cattle

A total of 1040 cattle were sampled and examined for trypanosomes using PCR at baseline collection. Overall, 26.6% were found to be infected with different trypanosome species including *T. brucei* s.l., *T. vivax*, *T. congolense* Savannah and mixed infections with overall prevalences of 20.8%, 5.7%, 0.2% and 1.7%, respectively. The human infective *T. b. rhodesiense* species was identified in 1.3% of the examined animals.

Age of the animal

Trypanosome prevalence in cattle was shown to increase with age, with the highest prevalence found in the adult age group (31.1%) when compared to the 1.5-3 years age category (25%) and the young age group (20.9%). Looking at the individual species, infections of *T. brucei* s.l. prevalence significantly increased with age with 24.5% in adults, 17% in 1.5-3 years age category and 7.8% in young calves. These findings agree with Kalu (1995) and Magona *et al*. (2004) who stated that cattle aged 6-9 years are more likely to be infected than younger animals. This might be attributed to the increased exposure to infected tsetse with age (Magona *et al.*, 2000; Rowlands *et al.*, 1993), only 10% of tsetse were reported to be feeding on year-old calves compared to 50-60% on adults (Torr and Mangwiro, 2000). The authors attributed this preference to the highly developed defensive mechanism in young animals against tsetse bites, while, the flies are more attracted to the larger and calmer adults. Moreover, host odours play an important role in the relative attractiveness of cattle to tsetse flies. In particular, young animals produce less odour stimuli that attract tsetse to the host, the odour of 500 kg ox is about twice as attractive as a 50 kg calf (Torr *et al.*, 2006).

In contrast to *T. brucei* s.l.; *T. vivax* were found to be significantly higher in young calves (11.3%) compared to 6.1% and 4.5% prevalence in 1.5-3 years age group and adults, respectively. This could be attributed to the short lifecycle of *T. vivax* in tsetse flies and the mechanical transmission with other Tabanids leading to the rapid appearance of *T. vivax* than *T. brucei* s.l. in young age (Dale *et al.*, 1995; Desquesnes and Dia, 2003). However, with increased age, *T. brucei* s.l. becomes more predominant than *T. vivax* which might be explained by a development of a sort of immunity in animals against *T. vivax* infection with age (Bourn and Scott, 1978; Rowlands *et al.*, 1993). Another explanation could

be death of calves due to infection with the highly pathogenic *T. vivax* leading to removal of the animals from the population.

Gender of animals

No statistical significant difference was observed in the prevalence of the overall parasitic events and infections with *T. brucei* s.l. between male and female cattle in the current study. These findings were in agreement with other studies that reported the insignificant influence of animal gender in the prevalence of trypanosomes (Kalu, 1995; Katunguka-Rwakishaya, 1996; Wissmann, 2007). The only significant difference in the study was observed in the prevalence of *T. vivax* where male cattle were found to be infected with 7.3%, while, female cattle had only 3.8% prevalence. This might be due to the larger size of male cattle that causes more attraction for biting insects (Rowlands *et al.*, 2001; Torr *et al.*, 2006; Torr and Mangwiro, 2000).

Body condition of the animal

The difference in the overall parasitic events prevalence between the lean, medium and fat animals was significant. The prevalence in lean animals (38.9%) was significantly higher than that in fat animals while insignificantly higher than the prevalence in medium scored animals (28.5% and 12.1%, respectively).

The prevalence of *T. brucei* s.l. infections was significantly higher in lean animals (27.8%) compared to 6.1% prevalence in fat animals. However, infections with *T. brucei* s.l. was lower in medium scored animals (21%) compared to lean animals and this was statistically insignificant. The insignificant difference between lean and medium scored animals in the prevalence of overall parasitic events and *T. brucei* s.l. indicated that condition score is not a good indicator for the presence of trypanosome infection (Wissmann, 2007). This conclusion was supported by the insignificant difference in infections with *T. vivax* between condition scored animals, as *T. vivax* is more pathogenic to cattle than *T. brucei* s.l.

Animal breed

It is estimated that almost all (more than 95%) of cattle in Uganda are of Zebu or Sanga types (Ankole breed) (Magona and Mayende, 2002). The Ankole breed have been brought to the country recently as a result of a re-stocking programme (Fevre *et al.*, 2001; Magona and Mayende, 2002; Magona *et al.*, 2004). The most predominant breed of cattle in the current study was Zebu cattle (87.4%) while only 7.9% were of the Ankole breed. The results showed a significantly higher prevalence of the overall parasitic events in the Zebu breeds (30%) than the 17.9% prevalence in the Ankole breeds. Moreover, Zebu cattle were infected with *T. vivax* while none of the Ankole cattle were found to be infected with this species.

Regarding *T. brucei* s.l. infections, there was no statistical significant difference between Zebu and Ankole cattle with 21.8% prevalence in Zebu cattle and 16.7% in Ankole. These findings were not in agreement with the previously reported susceptibility of Ankole cattle to trypanosome infection (Magona *et al.*, 2004; Waiswa and Katunguka-Rwakishaya, 2004). This could depend on how long the Ankole breeds had been in the infested area after re-stocking from trypanosome free area. However, Zebu cattle were reported to be more susceptible to trypanosome infection than Friesian and N'Dame cattle (Magona and Mayende, 2002; Naessens, 2006).

6.5.1.2 Prevalence of different trypanosome species at district level

The results in Table 6.4 and Figure 6.4 show that at baseline, infection with *T. brucei* s.l. significantly dominates the other species in the studied districts with an overall prevalence of 20.8%. *T. brucei* s.l. infection was followed by *T. vivax* and *T. congolense* Savannah with respective prevalence of 5.7% and 0.2%.

Table 6.8 summarises the reported prevalence of infections with different trypanosome species in cattle sampled from different parts of Uganda with reference to the method used in diagnosis.

Table 6.8: Reported prevalence of infections with different trypanosome species in cattle, Uganda

Area	Sample size	Methodology of diagnosis	*T. brucei* s.l.	*T. vivax*	*T. congolense*	Mixed infection (Type)	Reference
Central (Kampala)	486	Parasitological	14.8%	2.1%	0	2.1% *(T. brucei/T. vivax)*	(Clausen *et al.*, 1998)
Eastern (Soroti)	1475	Parasitological	1.4%	5.6%	0.6%	0.8% (not reported)	(Magona *et al.*, 2004)
South-western	1309	Parasitological	0	5.4%	0.8%	0.2% (*T. vivax/T. congolense*)	(Waiswa and Katunguka-Rwakishaya, 2004)
South-eastern (Buvuma islands)	59	Parasitological	10.2%	5.1%	1.7%	1.7% (*T. brucei/T. vivax*)	(Magona *et al.*, 1999)
South-eastern	3344	Parasitological	5%	5.4%	2.1%	0.7% (0.5% *T. brucei/T. vivax*, 0.2% *T. brucei/T. congolense*)	(Waiswa *et al.*, 2003)
South-eastern	1992	Parasitological	0.9%	4.2%	4.2%	1.5% (0.6% *T. brucei/T. vivax*, 0.9% *T. vivax/T. congolense*)	(Magona *et al.*, 2005)
South-eastern	1419	PCR	9.7%	4.9%	0.7%	0	(Fyfe, 2007)
Central	1040	PCR	20.8%	5.7%	0.2%	1.7%	Current study

Comparing the results obtained in the current study with those previously reported in different parts in Uganda, *T. brucei* s.l. prevalence in the current study was higher than those previously reported (Table 6.8). This higher prevalence might be attributed to the use of the highly sensitive molecular techniques for the detection of trypanosomes and the copy number of the PCR targets, while, other studies in the table (except Fyfe, 2007) used parasitological methods. Although in the study conducted by Fyfe (2007) molecular diagnosis was also used but the prevalence of *T. brucei* s.l. was lower than that obtained in the current study. This could be due to the different areas where studies were conducted or due to the use of single FTA disc for PCR reactions in Fyfe's study. In the current work DNA was eluted using Chelex®100 from 10 discs which increased the chances of amplifying trypanosome DNA.

In the current work, the prevalence of *T. b. rhodesiense* at baseline was 1.3% within the total sample population. This percentage is lower than *T. b. rhodesiense* reported in Uganda in other studies. An overall prevalence of 2.5% was reported in cattle sampled and examined by SRA-PCR in south-eastern Uganda by Fyfe (2007), this prevalence is almost significantly higher than the obtained prevalence in the current study (χ_1^2=3.9, p=0.05). However, 11.5% cattle were diagnosed to be positive for *T. b. rhodesiense* in south eastern Uganda (Enyaru *et al.*, 2006; Welburn *et al.*, 2001).

In the current study, Dokolo district had a significantly higher prevalence of *T. b. rhodesiense* (2.8%) compared to 0.3% in Lira. The prevalence in Kabermaido in the current study was 0.3% which is lower than 3.3% prevalence detected in the same district in the study conducted by Enyaru *et al*. (2006). This might be due to the different sites examined or the use of different primers targeting the SRA gene that were developed by Radwanska *et al*. (2002).

Epidemiological theory, based on mathematical modelling, predicts that the prevalence of *T. b. rhodesiense* will exceed that of *T. b. brucei* in non-humans with three and 3.5 fold more prevalence of *T. b. rhodesiense* in the cattle and vector population, respectively (Rogers, 1988). In the current study, this was not the case, in contrast, *T. b. brucei* was observed to be far more prevalent in cattle host. Coleman and Welburn (2004) collected data about the prevalence of *T. b. rhodesiense* and *T. b. brucei* in non-humans, they reported that the relative prevalence of *T. b. rhodesiense* to *T. b. brucei* was 0.33 suggesting the occurrence of *T. b. rhodesiense/T. b. brucei* in 1:3 ratio. The discrepancy between theoretical predictions and empirical observations was further investigated and it was hypothesised that there are fitness costs associated with human serum-resistance when *T. b. rhodesiense* is not in the human host (Coleman and Welburn, 2004). Fitness is defined as the overall ability of an organism to survive and reproduce (Durso *et al.*, 2004). Comparing the relative prevalence of *T. b. rhodesiense* to *T. b. brucei* (P_{Tbr}/P_{Tbb}) in cattle examined in Uganda with the 0.06 value in the current study, Table 6.9 summarises the reported observations (Coleman and Welburn, 2004).

Table 6.9: Relative prevalence of *T. b. rhodesiense* to *T. b. brucei* in cattle

Methodology of diagnosis	Number of *T. b. rhodesiense*	Number of *T. b. brucei*	P_{Tbr}/P_{Tbb}	Reference
RFLP	15	46	0.33	(Hide *et al.*, 1996)
BIIT	4	6	0.67	(Mwambu and Mayende, 1973)
Isoenzyme analysis	14	42	0.33	(Gibson and Wellde, 1985)
BIIT	24	109	0.12	(Robson *et al.*, 1972b)
BIIT	39	93	0.42	(Waiswa *et al.*, 2003)
PCR	18	27	0.66	(Welburn *et al.*, 2001)
PCR	10	79	0.13	(Enyaru *et al.*, 2006)
PCR	101	244	0.4	(Fyfe, 2007)
PCR	14	216	0.06	Current study

The results in Table 6.9 show that the P_{Tbr}/P_{Tbb} reported in the current study (0.06) was lower than the previously reported ratio. This might be attributed to the sampling of cattle in the aforementioned studies from endemic sleeping sickness foci where *T. b. rhodesiense* was identified with more frequency than in the current study. However, the current study agrees with the published results in the predominance of *T. b. brucei* over *T. b. rhodesiense* supporting the fitness cost hypothesis reported by Coleman and Welburn (2004).

The prevalence of *T. vivax* was similar to that reported by Magona *et al*. (1999; 2004), Waiswa *et al*. (2003) and Waiswa and Katunguka-Rwakishaya (2004) in eastern, south-eastern and south-western Uganda, respectively. However, in Kampala, Clausen *et al*. (1998) identified only 2.1% to be infected with *T. vivax* using microscopical methods.

The prevalence of *T. congolense* Savannah in the current study was 0.2% which was lower than other identified species. Although this result is lower than the reported studies as shown in Table 6.8 generally, the prevalence of *T. congolense* Savannah in Uganda is low, ranging from 0-2.1% except for one study conducted by Magona *et al*. (2005) who reported that *T. congolense* was identified in 4.2% of the parasitological examined cattle, which might be attributed to mis-calssification of other trypanosomes as *T. congolense* using microscopy.

Mixed infections were identified either by parasitological or molecular methods in the reported studies summarised in Table 6.8. In the current study, the prevalence of *T. brucei* s.l./*T. vivax* mixed infection was 1.7%. As presented in Table 6.8 the same result was obtained by Magona *et al*. (1999) in south-eastern Uganda (Buvuma islands, Lake Victoria). Lower findings of mixed *T. brucei* s.l./*T. vivax* infection were reported in south-eastern Uganda by Waiswa *et al*. (2003) and Magona *et al*. (2005) with 0.5% and 0.6%, respectively, which is explained by different diagnosis techniques and different locations of the studies. No mixed infection with *T. brucei* s.l./ *T. congolense* Savannah or *T. vivax/T. congolense* Savannah were diagnosed in the current study, however, *T. vivax/ T. congolense* Savannah mixed infections were reported in south-eastern Uganda by Magona *et al*. (2005) and in south-western Uganda by Waiswa and Katunguka-Rwakishaya (2004) with 0.9% and 0.2% prevalence, respectively. Moreover, *T. brucei* s.l./*T. congolense* mixed infections were reported in south-eastern Uganda with

0.2% by Waiswa *et al*. (2003). The identification of the aforementioned mixed infections in the reported study areas while the absence of such mixed combinations in the current study might be attributed to the different sites studied or artefacts of the parasitological methods in the visual differentiation of trypanosomes.

The dominance of infections with *T. brucei* s.l. over other species was reported in the current work in all study areas. This dominance was mirrored in other results reported in Uganda by Clausen *et al*. (1998), Magona *et al*. (1999) and Fyfe (2007). In contrast, other studies conducted by Magona *et al*. (2004 and 2005) reported the dominance of *T. vivax* over other trypanosome species as mentioned in Table 6.8. This might be attributed to the use of parasitological diagnosis, while, the results reported by Fyfe (2007) showed dominance of *T. brucei* s.l. using PCR.

The results were broken down to the district level to observe any differences in trypanosome prevalence between the three districts included in the study. The overall parasitic events prevalence was significantly lower in Kabermaido than in Dokolo and Lira districts. Looking at the species separately, infections with *T. brucei* s.l. were significantly higher in Dokolo than in Lira and Kabermaido. The results obtained in Kabermaido of *T. brucei* s.l. prevalence were 16.9% which nearly agrees with the 19% prevalence from the same district in a study conducted by Amungi (2008) (using the same PCR reactions for the diagnosis of *T. brucei* s.l.).

As shown in the results summarised in Table 6.4, Dokolo district prevalence of *T. b. rhodesiense* was significantly higher than the other two districts. *T. vivax* prevalence was significantly higher in Lira; however, mixed infection prevalence was insignificantly higher in Lira than in Dokolo and Kabermaido.

6.5.2 Post intervention follow-up results

6.5.2.1 Impact of intervention at the three sampling time points across the study area

The impact of the treatment programme at the three sampling time points across the study area is summarised in Table 6.5 and Figure 6.7. The results show that a significant reduction of the overall parasitic events, *T. brucei* s.l., *T. vivax*, *T. b. rhodesiense* and mixed infection prevalence was observed three months post-intervention indicating the success of the campaign in the study area. Although *T. congolense* Savannah prevalence was also reduced at three months follow up, this drop was insignificant (due to the low number of infections at baseline). Nine months post-intervention the prevalence of the overall parasitic events, *T. brucei* s.l., *T. b. rhodesiense* and mixed infections was significantly higher than the prevalence at three months follow up, however, this increase was lower than the prevalence at baseline.

The ratio of *T. b. rhodesiense*/*T. b. brucei* at baseline was 1:15 which decreased three months post-intervention to 1:40 and then increased again to 1:16 nine months post-intervention. This finding indicated the re-infection of cattle with both *T. b. rhodesiense* and *T. b. brucei*.

In contrast, the prevalence of *T. vivax* infection at nine months post-intervention was significantly higher than that reported at baseline. This could be explained due to the short lifecycle of *T. vivax* which can be completed in just one week leading to rapid re-transmission of the parasite after the intervention or due to increased challenge of the animals with other vectors carrying *T. vivax* such as Tabanids (Dale *et al.*, 1995; Desquesnes and Dia, 2003).

The same trend was noticed in *T. congolense* Savannah prevalence; however, the effect of the treatment on prevalence was not significant. This was in agreement with those reported by Fyfe (2007) who showed that *T. congolense* Savannah was detected with 0.7% prevalence at baseline; no cases were detected at three months follow up. However, one year post-intervention, cases were detected again although the prevalence remained very low (0.2%).

The results in Table 6.5 show the prevalence of trypanosomes in animals that had not received any treatment. These animals could be used as a marker to estimate the impact of the intervention programme in the studied area. The prevalence of infection with *T. brucei* s.l., *T. b. rhodesiense*, *T. vivax* and *T. congolense* Savannah at three months follow up sampling was 9.6%, 0%, 2.4% and 0%, respectively. The prevalence of the aforementioned species was lower than the baseline correspondent prevalence which might indicate the decreased challenge of all the animals in the study area with tsetse flies due to the initial spraying coverage. However, at nine months follow up, *T. brucei* s.l., *T. b. rhodesiense*, *T. vivax* and *T. congolense* Savannah prevalence in the non treated animals was insignificantly different from the prevalence in animals subjected to intervention, indicating the increased challenge of all the animals with the flies due to inadequate spraying.

The type of mixed infections identified at baseline was *T. b. brucei*./*T. vivax* with no *T. b. rhodesiense* involved in the combination. No mixed infection was detected three months post-intervention. Nine months post-intervention, mixed infection combination included one *T. brucei* s.l./ *T. congolense* Savannah and two of the remaining 14 *T. brucei* s.l./*T. vivax* included *T. b. rhodesiense* sub species.

6.5.2.2 Impact of intervention at district level

The results obtained were broken down to district level to find out if there was any difference between the districts included in the current study. The results in Table 6.6 show that there was a significant reduction of the overall parasitic events three months post-treatment in the three districts, indicating the success of the mass treatment in reducing trypanosome species from animals. Although there was a significant increase of the prevalence nine months post-intervention (compared to three months), this increase was insignificantly lower than the baseline prevalence. So, in the absence of sustained control

measures the trypanosome population returned to the baseline level meaning that the transmission is not interrupted by single mass treatment. This also might be attributed to the lower proportion of sampled animals at three and nine months follow up that had received treatment due to slaughtering, selling or introduction of new animals.

Looking at the impact of the treatment programme on the prevalence of *T. brucei* s.l. in the three chosen districts, the results showed also a significant reduction three months post-intervention. Lira district had the significantly higher reduction in *T. brucei* s.l. infection than the other two districts. The intervention programme studied by Fyfe (2007) in south-east Uganda showed a significant reduction in *T. brucei* s.l. prevalence in Soroti and Kamuli districts three months following treatment. The human infective species *T. b. rhodesiense* was significantly reduced in Dokolo and Lira district post-intervention; however, in Dokolo the prevalence was significantly increased again by nine months post-intervention to prevalence insignificantly lower than the baseline prevalence.

Regarding *T. vivax* prevalence, a significant reduction was only noticed in Lira district at three months post-intervention. In contrast to *T. brucei* s.l., the re-infection of animals with *T. vivax* was higher than the prevalence at baseline. However, only Kabermaido district had a significant increase in *T. vivax* prevalence at nine months compared to baseline. This coincides with the results obtained by Fyfe (2007) who noticed a paradoxical increase of *T. vivax* prevalence in Soroti district (adjacent to Kabermaido district) one year post-intervention although this was statistically insignificant. Fyfe (2007) argued that the short development period of *T. vivax* within the vector might result in the rapid resumption of *T. vivax* transmission after any intervention.

6.5.2.3 Impact of intervention at village level

The results in Figures 6.11-6.13 show the impact of the treatment programme on the prevalence of trypanosomes at village level. A significant reduction of the overall parasitic events prevalence was detected in six villages including Omer, Awinyipany, Okwar, AbuongB, Ogengo and Onyakede villages. The impact of the campaign was insignificant in Mwatma and Abienek villages. Unexpectedly, although the proportion of animals in Olep village that had received treatment was 94.6%, the prevalence of the overall parasitic events was insignificantly higher at three months follow up compared to the baseline prevalence. Nine months post-intervention, the only village that had continous reduction of the overall parasitic events prevalence was Amwoma village. However, re-infection of the animals was reported in the other villages indicating the importance of continuous treatment of the animals accompanied with spraying to decrease the challenge with tsetse flies.

Infection with *T. brucei* s.l. species was significantly reduced three and nine months post-intervention in Omer and Mwatma villages indicating the success of the campaign in these two villages in clearing the infection for nine months post-intervention. Although a reduction in *T. brucei* s.l. prevalence was noticed in Mwatma village during the study period this reduction was insignificant. In Awinyiipany,

Okwar, AbuongB and Ogengo villages there was a significant reduction of *T. brucei* s.l. infections three months post-intervention.

The impact of the treatment programme on the infection with *T. vivax* species was only significant in AbuongB village at three months post-intervention.

T. congolense Savannah was only detected in Abieneik village at baseline and three months post-intervention. Nine months post-intervention, the infection with *T. congolense* Savannah was cleared from Abieneik village and detected in Mwatma and Awinyiipany villages. This could be due to movement of the animals or slaughtering of the animals that were infected in Abieneik and challenge of the animals at the other two districts with flies infected with *T. congolense* Savannah.

6.5.2.4 Impact of the drugs used on the prevalence of trypanosome species infections

Diminazine-aceturate is a curative trypanocidal drug without any reported protective period leading to re-infection of the treated animals if they were challenged with flies carrying trypanosomes. However, ISM has been reported to give a prophylactic period of 2-3 months when it is used at a dose of 1 mg/kg body weight (Peregrine *et al.*, 1991).

The results in Table 6.7 show the impact of using ISM and DI on the prevalence of different trypanosome species. The two drugs showed a significant reduction on the prevalence of the overall parasitic events, *T. brucei* s.l., *T. vivax* and mixed infections. However, the percentages of reduction of the aforementioned species prevalence were higher using DI than using ISM. It was expected that cattle in areas treated with DI would be re-infected with trypanosomes at the three months follow up; however, this was not the case, with the drug showing a significant reduction of trypanosome species.

Diminazine-aceturate was reported to clear the experimentally infected animals with *T. vivax* 24 hours following infection (Desquesnes, 1997). Moreover, the fast recovery of PCV in animals infected with *T. brucei* s.l. and *T. congolense* was reported by Van den Bossche *et al*. (2005) following DI treatment. The re-infection of animals with trypanosomes nine months post-intervention could be attributed to the insufficient spraying coverage, the absence of re-treatment coverage, re-infection from a tsetse and also the inability of the drugs to reach body tissues such as the brain where *T. brucei* s.l. is sometimes located (Waiswa *et al.*, 2003).

The appearance of new *T. vivax* infections nine months post-intervention in the current study using ISM and DI agrees with the reported results by Stevenson *et al*. (1995) who stated that *T. vivax* infections were not always cleared by ISM. They related the re-appearance of *T. vivax* infection following ISM treatment to resistance to the drug which has become increasingly common due to wide use of ISM to control trypanosomiasis in Africa (Stevenson *et al.*, 1995).

Drug resistance against ISM and DI has been reported in different parts of Africa due to regular use of these drugs in treatment and prophylaxis. Resistance against ISM has been reported in cattle in western Ethiopia (Tewelde *et al.*, 2004), while in Zambia resistance against both ISM and DI was reported by Sinyangwe *et al*. (2004). Development of DI resistance was reported by Rowlands *et al*. (1993) in Ethiopia after 20 days of treating the cattle with 7 mg/kg DI. Although in the current study relapse of trypanosomes was noticed at nine months follow up, three months post-intervention the reduction was significant.

The inconsistency in the findings of the current study and the results reported by Rowlands *et al*., (1993) might be due to different geographic locations. Moreover, experimental infection of cattle with *T. vivax* revealed the relapse of infection following treatment with DI and the authors argued that this relapse might have resulted from drug resistance (Bengaly *et al.*, 2001). However, drug resistance in Uganda against the two drugs has not been reported because these drugs are not used as regularly when compared to other parts of Africa. Although drug resistance in Uganda has not been reported, attention should be paid to avoid the unsupervised use of the limited range of trypanocidal drugs. This is because the unsupervised use of the limited range of drugs available for the treatment of trypanosomiasis could hasten the emergence of cross-resistant strains of trypanosomes, the control of which would be more difficult and more expensive (Connor and Halliwell, 1987).

In conclusion, the analyses of the results indicated the success of the mass treatment programme in reducing the prevalence of trypanosome species from animals three months post-intervention. The re-infection of the animal population with trypanosomes in absence of continued treatment as seen nine months post-intervention indicated high challenge with infected tsetse flies or possibly movement of infected animals into the study area. The results denote that in the absence of sustained intervention, the trypanosome level returned to pre-treatment levels, however with an altering of the dynamics of the population with *T. vivax* occurring more frequently. This might be attributed to the short lifecycle in the fly of *T. vivax* or increased challenge of animals with other vectors carrying *T. vivax*. However, anecdotal evidence suggested that animals included in this programme were seen to be healthier and more productive by their owners.

7 Chapter seven:

Case study two: Molecular studies of experimental mixed infections of *T. congolense* Savannah and *T. b. brucei* in *G. m. morsitans*

7.1 Introduction

7.1.1 Mixed trypanosome infections in tsetse flies

Natural infection rates in tsetse flies are traditionally estimated by microscopical examination of different parts of the fly after dissection, together with the isolation of the parasite *in vivo* (Hoare, 1972). These methods are laborious and time consuming, coupled with the potential inaccuracy of differentiating between immature infections of *T. brucei* s.l. and *T. congolense* and mature infections of *T. congolense* and *T. vivax*.

Field studies have revealed that many vertebrate species which serve as natural hosts of *Glossina* often carry mixed infections of *T. brucei* s.l., *T. congolense* species and/or *T. vivax* (Moloo *et al.*, 1982). Therefore, the frequency of mixed infections in wild flies depends on the frequency of mixed infections in mammalian hosts (Gibson and Ferris, 1992). Mixed infections might result from sequential infections (fly feeds on two infected animals) or simultaneous infections where the fly feeds on one animal with a mixed infection (Morlais *et al.*, 1998b).

Mixed species infections have been widely reported in tsetse flies with a variation in the prevalence; for instance, Woolhouse *et al*. (1996) reported only 6.2% prevalence of mixed infections in 10,000 tsetse flies collected from Zimbabwe and examined with PCR. Lower prevalence of mixed infections were documented in Cameroon by Morlais *et al*. (1998a) who identified 2.2% mixed infections in 943 tsetse flies using PCR. However, another study conducted in the same area reported a prevalence of 7.1% mixed infections in 467 tsetse flies examined by PCR analysis (Morlais *et al.*, 1998b).

In Tanzania, Adams *et al*. (2006) reported a prevalence of 1% mixed infections in 2392 tsetse flies using PCR on midguts applied on FTA®cards. In northern Tanzania, 3741 flies were examined and 3.4% of infections were documented to be of mixed species by Lehane *et al*. (2000) using PCR. In Kenya, 1.3% of the examined 3826 flies were harbouring mixed infections (Njiru *et al.*, 2004b). In West Africa, Cote d'Ivoir, mixed infections were reported in 2.5% of the examined 811 flies using PCR (Masiga *et al.*, 1996), however, a higher prevalence of 6.5% was reported in the same area by McNamara *et al*. (1995b).

It has been also shown that it is possible for tsetse to acquire mixed infections experimentally either simultaneously or sequentially, a number of studies have investigated the occurrence of mixed infections in experimentally infected flies using an end point PCR (Gibson and Ferris, 1992; Kubi *et al.*, 2005; Masiga *et al.*, 1992; Moloo *et al.*, 1982; Peacock *et al.*, 2007; Reifenberg *et al.*, 1997b). However, none of these studies has looked to quantify the infection load of tsetse flies with trypanosomes in these mixed infections. End point PCR is only qualitative and is time consuming due to the need for visualising the results by agarose gel. In contrast, although more expensive, qPCR

allows the quantification of DNA and is also less time consuming due to real time visualization of the results which in the long run can reduce the overall costs (Ranasinghe *et al.*, 2008).

As the natural infection rates of the University of Edinburgh tsetse colony range from 5 to 20% (MacLeod, 2005), it was decided, for preliminary work, to artificially increase the infection rates using glutathione (GSH). As this was a preliminary experiment to validate qPCR as an effective tool for the measurement of trypanosome populations in the tsetse midgut, it was felt that it would be better to give the trypanosomes every chance of establishing an infection (through the addition of GSH) rather than go down the natural infection route where many hundreds of flies would have to be dissected to gain similar numbers of infected midguts.

GSH is an antioxidant and is thought to mop up free radicals produced either by the fly or as part of the bloodmeal breakdown during the establishment phase in the midgut (MacLeod *et al.*, 2007). Previous studies had suggested that tsetse derived lectins were responsible for trypanosome death (Welburn *et al.*, 1993) as the addition of the sugar glucosamine could increase infection rates (Maudlin and Welburn, 1987). However, it has been recently recognised that glucosamine can act as an antioxidant (Xing *et al.*, 2006) and it may be that glucosamine acts in a similar way to GSH in mopping up free radicals (MacLeod *et al.*, 2007).

7.1.2 Quantitative PCR

7.1.2.1 Principle of qPCR

Different methods of DNA quantification are used in molecular biology such as PCR-ELISA and Southern hybridization. Although, the limitations of these methods include the fact they are time consuming, labour intensive, insufficient sensitivity, inaccurate for quantification, require the use of radioactivity and may result in a substantial probability of cross contamination (Reischl *et al.*, 2002; Valasek and Repa, 2005).

Recently, fluorescent real-time monitoring of amplicon accumulation has been developed to overcome the limitations encountered with conventional PCR reactions so that post-PCR end point analysis is not necessary (Heid *et al.*, 1996). Quantitative PCR has been found to have advantages over the aforementioned methods, most importantly in the ability of accurate quantification of the target DNA with high sensitivity, allowing the detection of less than five copies of the target sequence. Moreover, the rapid performance and the removal of the visualization stage using gel electrophoresis makes qPCR quicker and less liable to contamination because handling of the samples is minimized (Klein, 2002; Valasek and Repa, 2005).

Quantitative PCR was pioneered by Higuchi and colleagues (1992 and 1993) who included ethidium bromide, as a fluorescent dye. The PCR was run under ultra-violet (UV) light thus causing the

ethidium bromide to fluoresce; researchers were able to visualise and record the accumulation of DNA with a video camera (Higuchi *et al.*, 1992; Higuchi *et al.*, 1993). The combination of high sensitivity and specificity, low contamination risk, ease of performance and speed, has made qPCR technology an appealing alternative to conventional methods (Espy *et al.*, 2006).

The quantification of the template original copy number is determined at the exponential phase of the reaction; at this point the reaction is able to efficiently amplify the DNA in the presence of adequate reactants (Figure 7.1). However, during the plateau phase, no more products are amplified due to the exhaustion of the ingredients and if left long enough, the PCR products will begin to degrade (Newton and Graham, 1997; Valasek and Repa, 2005). The crucial conceptual innovation and the key to understanding quantification by qPCR is the threshold cycle (Ct), which is the cycle at which the amplification plot crosses a fluorescence threshold (Bustin, 2006) (Figure 7.1). The Ct value is inversely proportional to the amount of the specific nucleic acid sequence in the original sample.

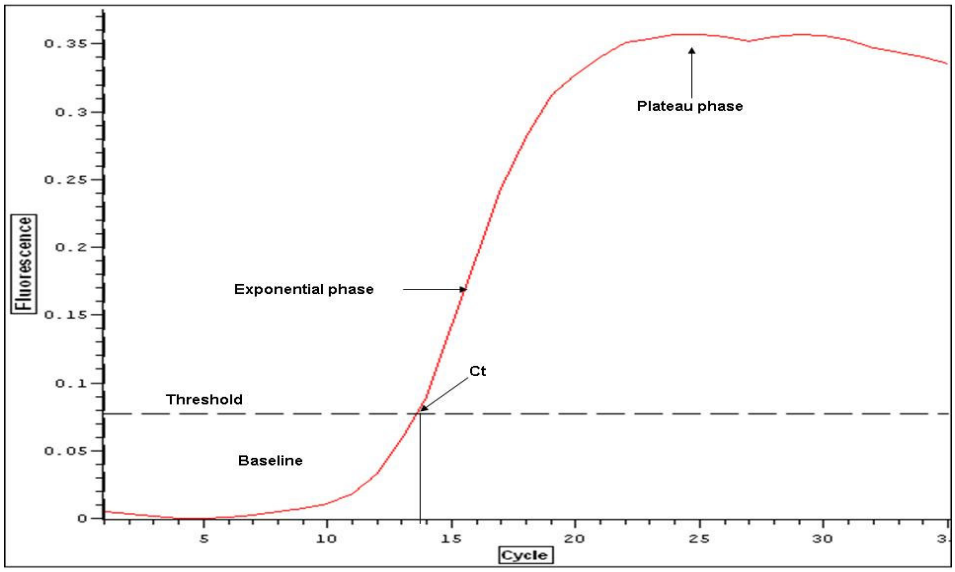

Figure 7.1: A diagram showing the crossing of the fluorescence plot to a fluorescence threshold to determine Ct value for each sample during PCR amplification

Although the advantages achieved by using qPCR are plentiful, several limitations are also encountered, including the inhibition of qPCR by compounds that occur in certain types of samples, for example, samples from body fluids contain inhibitors such as urea and haemoglobin (Valasek and Repa, 2005; Wilson, 1997). This limitation may be circumvented using alternative DNA polymerases (*Pwo, Tth*) that are resistant to particular inhibitors. Another major limitation is the use of RNA in gene expressions assays because RNA is more labile than DNA, therefore, isolation should be carefully performed to ensure both the integrity of RNA itself and the removal of contaminating

nuclease genomic DNA and PCR inhibitors (Bustin, 2002; Bustin and Nolan, 2004; Valasek and Repa, 2005).

7.1.2.2 Chemicals required for qPCR

Monitoring the progress of DNA amplification in real-time requires specific chemicals and instruments. The required chemicals for qPCR consist of special fluorescent reporters; different types of fluorescent reporters are currently used for qPCR such as SYBR Green I, 5'-nuclease probes (TaqMan), molecular beacons and fluorescence resonance energy transfer (FRET) hybridization probes (Espy *et al.*, 2006; Valasek and Repa, 2005).

SYBR Green I

SYBR Green I is an intercalating dye that binds to double stranded DNA emitting fluorescence that is 1000-folds higher than when it is free in solution (Valasek and Repa, 2005; Wittwer *et al.*, 1997). Therefore, the amount of fluorescence increases proportionally with the amount of DNA (Figure 7.2).

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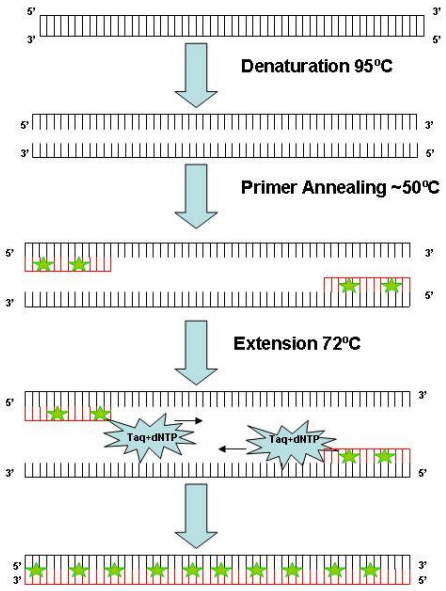

Figure 7.2: Illustration of SYBR Green I mechanism of binding (green stars represent SYBR Green I molecules), protocol adopted from Valasek and Repa (2005)

Due to the higher sensitivity of the dye, it will bind to any double stranded DNA occurring in the tube, so that in addition to the desired target, primer dimers and other extraneous double stranded products

may also be detected (Valasek and Repa, 2005; Wittwer *et al.*, 1997). In this case, the dissociation curve of the amplified product is analysed to determine the melting temperature that gives an indication if one or more products were amplified allowing us to distinguish between the amplified products and primer dimers or non-specific amplicons (Figure 7.3). Therefore, once the protocol has been optimised there should be no problem with dimer formation.

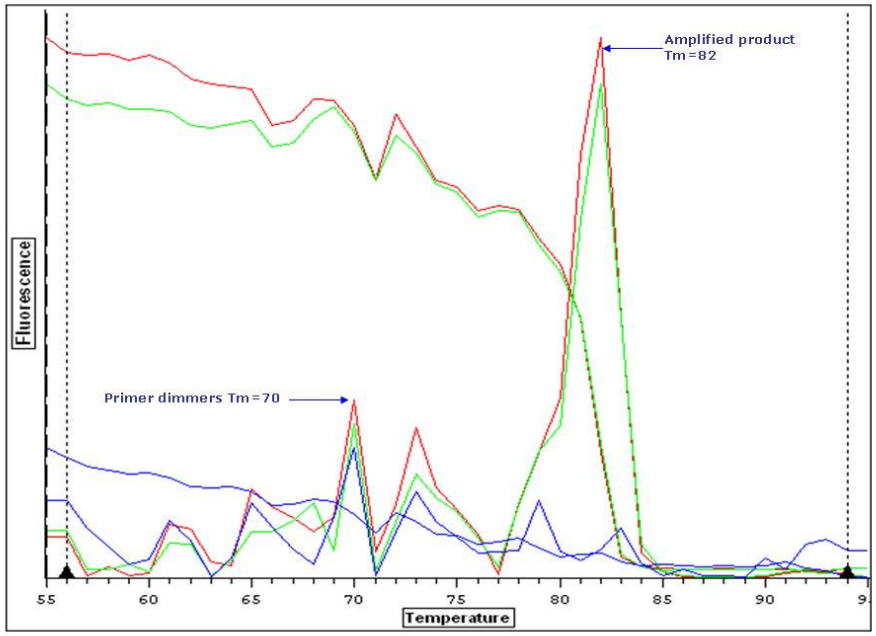

Figure 7.3: Dissociation curve showing the amplified product melting temperature (82ºC) and a second peak at lower melting temperature (70ºC) indicating primer dimers

Probes

For sensitive and specific detection of amplified products, the use of FRET probes that rely on the transfer of light energy between two adjacent dye molecules is currently accomplished by qPCR technology. The intensity of emitted fluorescence using FRET probes depends on the distance between the dye molecules, as the distance increases, FRET decreases (Espy *et al.*, 2006). The commonly used FRET probes are hydrolysis probes (TaqMan), molecular beacons and FRET hybridization probes.

TaqMan probes were the first developed 5'-nuclease probes for qPCR. They are a short DNA sequence (single strand) that are designed to be complementary to a specific sequence in the target; it contains a fluorescent dye (reporter) at the 5' end and a quenching dye (absorbs the signal from the fluorescent dye) at 3' end. The light of the fluorescent dye is not emitted as long as the quencher is attached to the probe, in order to remove the effect of the quenching dye to generate the light signal,

firstly the probe must bind to the complementary sequence at 60ºC. Secondly, at the same temperature, the *Taq* polymerase cleaves (free) the 5' end of the probe separating the fluorescent dye from the quenching dye, causing accumulation of the fluorescent dye emitting the light signal which can be measured at any time during the PCR cycle including the hybridization step (Figure, 7.4). FAM, VIC, NED are examples of the fluorescent dyes and TAMRA, DABCYL and BHQ are examples of quencher dyes (Espy *et al.*, 2006; Mackay, 2004; Mackay *et al.*, 2002).

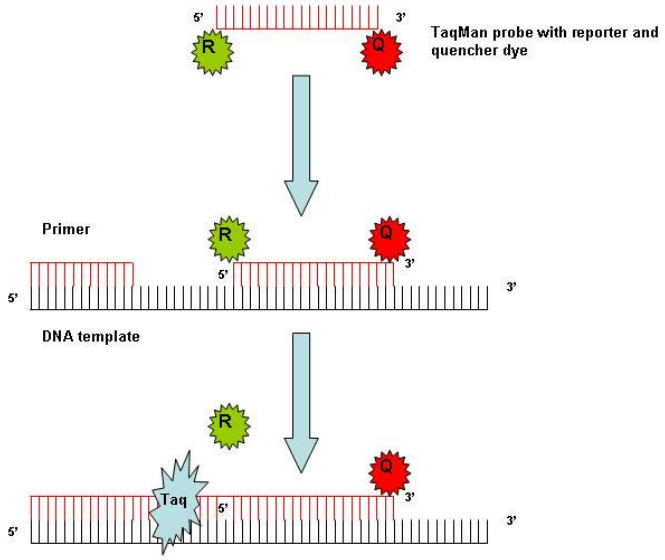

Figure 7.4: TaqMan probe composition and mechanism of action (R: reported dye, Q: quencher dye), protocol adopted from Espy *et al*. (2006)

Molecular beacons are another type of fluorescent probes used in qPCR, based on a sequence that binds to a complementary part in the target DNA. A fluorescent reporter is attached to the 5' end and the quencher is attached to the 3' end; in contrast to TaqMan probes, the reporter is not cleaved by the 5' nuclease activity. The mechanism of action of molecular beacons (Figure 7.5) depends on the design of the probe with a region at each end to be complementary to each other, so at low temperature, the ends anneal creating a hairpin structure. This close proximity leads to quenching of the fluorescence from the reporter dye, as the temperature increases, the probe and the target are single stranded and hybridization of the probe to the complementary sequence occurs leading to separation of the reporter from the quencher omitting light signal which can be measured at the time of hybridization only (Espy *et al.*, 2006; Mackay, 2004; Mackay *et al.*, 2002).

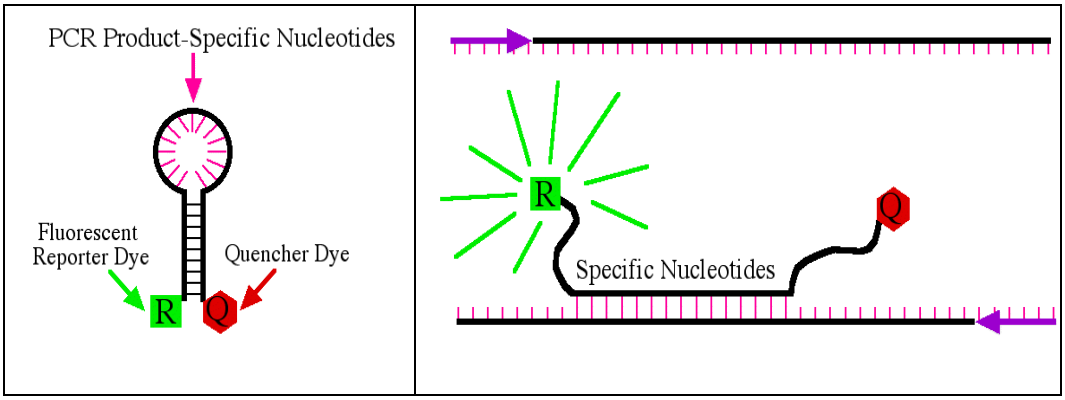

Figure 7.5: Molecular beacon before (left) and after (right) hybridization to the specific target (www.bio.davidson.edu/Courses/genomics/method/beacon1.gif)

Finally, FRET hybridization probes are two DNA probes designed to anneal next to each other in a head-to-tail configuration on the PCR product. The upstream probe has a fluorescent dye on 3' end and the downstream probe has an acceptor dye on the 5' end. If both probes anneal to the target, fluorescence from the 3' dye is absorbed by the adjacent acceptor dye which when excited will emit light to be detected by the qPCR machine (Figure 7.6). In the absence of target, the probes will not anneal and the two dyes will not associate with each other thus preventing FRET from occurring. The 3' end of the downstream probe is phosphorylated to prevent it from being used as a primer by *Taq* polymerase during PCR amplification. The specificity of FRET hybridization probes is extremely high because the two probes encompass a region of 40-50 bp (Espy *et al.*, 2006; Mackay, 2004; Mackay *et al.*, 2002).

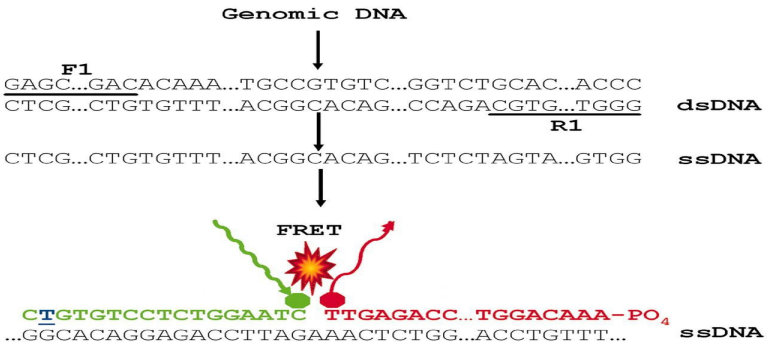

Figure 7.6: Illustration showing the mechanism of action of FRET hybridization probes (acceptor dye in red and fluorescent dye in green)

(nar.oxfordjournals.org/content/vol32/issue7/images/large/gnh059f1.jpeg)

7.1.2.3 Instruments

An important requirement for qPCR technology is the use of specific instruments able to detect the fluorescent signal and record the progress of the PCR. Fluorescent chemicals require both a specific input of energy for excitation and a detection of a particular emission wavelength; for these purposes, the instrumentation should be able to do both simultaneously at a desired wavelength. Quantitative PCR machines can supply the excitation energy using a lamp, light-emitting diode (LED) or laser, the desired wavelength is filtered to the sample. The emission energies are filtered to obtain the appropriate wavelengths and detected using photo-detectors, such as cameras, which transfer the data to data-acquisition and analysis software. Figure 7.7, shows the basic instruction of qPCR instrument (Espy *et al.*, 2006; Kubista *et al.*, 2006; Valasek and Repa, 2005).

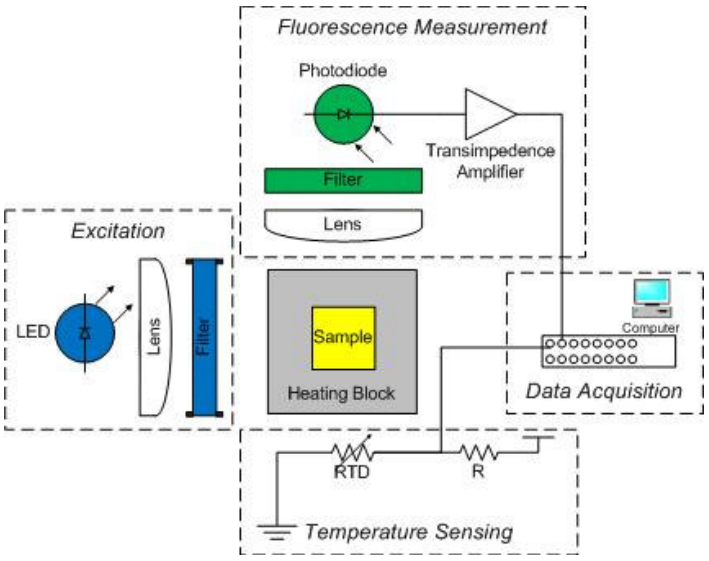

Figure 7.7: Illustration of the basic structure of qPCR instrumentation

(openwetware.org/images/3/31/DNA_Melting_Block_Diagram.jpg)

7.1.2.4 Absolute and relative quantification

Absolute quantification is used to determine the copy number of a certain gene or the load of infective pathogen in a sample; this is achieved by generating a standard curve from dilutions of a known standard. Running unknown samples in the same assay with the standard curve quantifies the DNA in those samples when their Ct values are compared with the standard curve (Kuhne and Oschmann, 2002; Valasek and Repa, 2005). Relative quantification measures changes in the steady-state levels of the gene of interest relative to control genes (housekeeping gene) such as ß-actin and 18S rRNA (Dheda *et al.*, 2004; Valasek and Repa, 2005).

7.1.2.5 Application of qPCR in trypanosomes and insect vectors

The use of qPCR in quantifiying *T. brucei* s.l. in human blood samples was first reported by Becker *et al*. (2004). The authors developed a qPCR assay for the detection of *T. brucei* s.l. in blood samples from sleeping sickness patients collected onto FTA®cards. DNA was eluted from 10 discs using Chelex®100 and subjected to qPCR reaction. The authors re-designed the primers targeting the 177 bp repetitive DNA satellite sequence (Moser *et al.*, 1989a) to get rid of the tendency of the primers to form secondary structures. The reported sensitivity of the reaction was 100 trypanosomes/ml blood, with an efficiency of 86%.

In the insect vector, qPCR has been used to estimate *Leishmania chagasi* load in experimentally infected sand fly vectors (Ranasinghe *et al.*, 2008). The authors used a TaqMan qPCR that had an efficiency of 75% with a sensitivity of 120 parasites. Also, qPCR reaction based on SYBR Green I chemistry was reported to have efficiency of 88.6% and a sensitivity of detecting 10 *Plasmodium falciparum* parasites in experimentally infected mosquito vector (Bell and Ranford-Cartwright, 2004).

7.2 Objectives

The aim of the work presented in this chapter is to evaluate qPCR as a tool for the quantification of *T. b. brucei* and *T. congolense* Savannah in tsetse midgut infection. Moreover, it aims to study differences in the number of each trypanosome species in the midgut of the fly in mixed infections.

7.3 Material and Methods

7.3.1 Quantitative PCR

QPCR was performed in a DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research), each set of reactions are able to assay 96 tubes. Standards and samples were tested in triplicate and expressed as mean values. Controls consisted of tubes containing the mastermix only (no template DNA) were also used.

7.3.1.1 Preparation of trypanosome procyclic forms

Procyclic forms were prepared as previously explained in Chapter IV (sections, 4.3.1.1 and 4.3.1.2). The number of the trypanosomes per ml was estimated using haemocytometer quantification.

7.3.1.2 Preparation of standards for qPCR

Standards for qPCR quantification were prepared using a ten fold serial dilution from the procyclic cultures after quantification of *T. b. brucei* and *T. congolense* Savannah using a haemocytometer to

obtain standard ranging from $4x10^6$ to 4 parasites per ml, as the DNeasy®Blood and Tissue kits (Qiagen), used to prepare the DNA, recommended a concentration $<5x10^6$ cells/ml. Cultured procyclics were centrifuged for five minutes at 300 x g, the pellet was then resuspended in 200 µl PBS and 20 µl proteinase K were added and the extraction was done as previously explained in Chapter III (section, 3.4.3.2).

In order to confirm the accurate quantification of the standards using the haemocytometer, DNA concentration was measured after extraction using a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE), and the concentration was related to the assumed amount of DNA from one parasite which is reported to be 0.1 pg (Borst *et al.*, 1982). After extraction of the DNA from *T. b. brucei* and *T. congolense* Savannah cultures, DNA concentration was measured and found to be $4.18x10^5$ pg and $4.13x10^5$ pg, respectively, these concentrations are equivalent to DNA from $4.18x10^6$ and $4.13x10^6$ parasites, respectively.

Trypanosome standards were spiked over a fixed amount of *Glossina* DNA to ensure that the latter was not an inhibitory factor in the reaction and to mimic natural conditions in the infected flies where trypanosome DNA exists with *Glossina* DNA. Moreover, the standards were spiked with the fly DNA to ensure that the increase in the fluorescence in the standards resulted from the increase of trypanosome DNA concentration. The amount of DNA to spike the standards was calculated according to the average *Glossina* DNA in a single fly. Using the NanoDrop quantification of the *Glossina* DNA in the non-infected midguts, the average amount of DNA was 2600 ng/fly (13 ng/µl). However, using the *Glossina* qPCR on non-infected flies, the average amount of the DNA in a single fly was 2724 ng/fly (13.6 ng/µl). The amount calculated using *Glossina* qPCR was added to each trypanosome DNA standard.

The *Glossina* DNA standards used for *Glossina* qPCR were prepared by 10 fold serial dilution of the DNA starting from a concentration of 93.4 ng/µl and four points of dilution were used.

The cycle threshold of the reactions was detected by the Opticon software. The mean Ct and standard deviations were calculated for each individual standard using data from the triplicates, and graphs of mean Ct against standard concentration were plotted to obtain a line of best fit.

7.3.1.3 Evaluation and optimisation of *T. brucei* s.l. qPCR reactions

7.3.1.3.1 *T. brucei* s.l. qPCR using satellite DNA target

QPCR amplifying 177 bp repetitive DNA sequence of *T. brucei* s.l. was reported by Becker *et al.* (2004) using SYBR Green I PCR core reagents kit (Applied Biosystems). The authors re-designed the primers to overcome 6ºC difference between the melting temperatures of the two primers. The sequences of the primers are Tb177F: 5'-AAC AAT GCG CAG TTA ACG CTA T-3' and Tb177R: ACA TTA AAC ACT AAA GAA CAG CGT TG-3', these primers amplify 134 bp amplicon.

The amplification mixture contained 1 µl DNA template, 300 nM of each primer, 0.25 units AmpliTaq Gold DNA polymerase, 0.1 units AmpErase [uracil-N-glycosylase (UNG)], 0.2 mM of the four dNTPs, 0.4 mM dUTP, 3 mM $MgCl_2$ and 1 µl 10X SYBR Green I buffer with ROX fluorescence dye as a passive reference in a final volume of 10 µl.

The qPCR reaction was performed in a DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research). The reaction conditions used were 50ºC for two minutes to activate UNG, 95ºC for 10 min then 30 cycles at 94ºC for 20 seconds and 66ºC for one minute followed by plate read for fluorescence acquisition. A temperature gradient between 60ºC and 95ºC was run to obtain the dissociation curve. Amplicons were visualised using 1% agarose gel to confirm specificity of the reaction. The standards and samples were run in triplicates and expressed as a mean value, non-template controls were also used to check the presence of contamination.

7.3.1.3.2 *T. brucei* s.l. qPCR using a single copy gene target

Primers targeting the single copy glycosyl-phosphatidylinositol-specific phospholipase C (PLC gene) (Mensa-Wilmot *et al*., 1990) specific for *Trypanozoon*, reported by Picozzi *et al*. (2008) were used for qPCR. The sequences of the primers are PLC1: 5'- CGC TTT GTT GAG GAG CTG CAA GCA-3' and PLC2: 5'- TGC CAC CGC AAA GTC GTT ATT TCG-3', they amplify 324 bp amplicon.

The amplification mixture contained 1 µl DNA template, 200 nM of each primer, 5 µL SYBR Green I ready-made master mix supplied by Qiagen (containing HotStarTaq DNA Polymerase, Quantitect SYBR Green I PCR Buffer [Tris-Cl, KCl, $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, pH 8.7], 0.2 mM dNTP mix, SYBR Green I dye, ROX dye), 0.1 units AmpErase [Uracil N-glycosylase] (Applied Biosystems). Water was added to a final volume of 10 µl.

The reaction conditions used were 50ºC for two minutes to activate UNG, 95ºC for 15 min then 40 cycles at 94ºC for 30 sec, 63ºC for 90 min and 72ºC for 70 sec followed by plate read for fluorescence acquisition. A temperature gradient between 55ºC and 95ºC was run to obtain the dissociation curve. Amplicons were visualised using 1% agarose gel to confirm specificity of the reaction. The standards and samples were run in triplicates and expressed as a mean value, non-template controls were also used to check the presence of contamination.

7.3.1.4 Optimisation of *T. congolense* Savannah qPCR

Primer optimisation

Two primer sets that had been previously used for the amplification of *T. congolense* Savannah (Masiga *et al.*, 1992; Moser *et al.*, 1989a) were chosen for the qPCR reaction in the current study. The primer sets were those amplifying a satellite DNA sequence which occurs in the parasite genome as

5400 copies and results in amplification of 316 bp of the product. However, according to the guidelines for primer design to be used in qPCR, the theoretical melting temperature of the two primers should be within 2ºC of each other. For this reason, one base was removed from the reverse primer sequence to obtain 2ºC difference. The sequences for the *T. congolense* Savannah primers used for qPCR are TCS-RF: 5'-GGA CAA ACA AAT CCC GCA CA-3' and TCS-RR: C GAG AAC GGG CAC TTT GCG A-3', these primers amplified 315 bp of the target sequence (the red colored base was the one removed from the re-designed primers).

For SYBR Green I chemistry, relatively low primer concentrations ranging from 50-300 nM are used to avoid primer-dimer formation, therefore, a primer optimisation matrix was used and for each primer pair; reactions were run in duplicate with the appropriate negative controls for each concentration. The ideal primer pair that resulted in lowest average Ct and a single product for the template when running a dissociation curve was chosen for the reaction.

Reaction conditions

Different SYBR Green I master mixes were used (Qiagen, Bio-Rad and Applied Biosystem), Bio-Rad kits was chosen because it resulted in lower Ct values. Each PCR reaction contained 1 µl of the extracted DNA, 300 nM of each primer, 5 µl iQ™SYBR®Green I supermix (Bio-Rad) that contains 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM of each dNTP, 0.25 units iTaq DNA polymerase, 3 mM $MgCl_2$, SYBR Green I, 10 nM fluorescein. Water was added to a final volume of 10 µl.

The cycling programme was initiated with denaturation of DNA and polymerase activation at 95ºC for 10 min followed by 30 cycles each of 94ºC for 20 sec and 60ºC for one minute followed by plate read for fluorescence acquisition. Dissociation curve was acquired by melting the amplified product from 55ºC to 95ºC. Amplicons were visualised using 1% agarose gel to confirm specificity of the reaction. The standards and samples were run in triplicates and expressed as a mean value; non-template controls were also used to check for the presence of contamination.

7.3.1.5 Optimisation of *Glossina* species qPCR

7.3.1.5.1 Preparation of *Glossina* DNA

DNA extraction from midguts was done using DNeasy®Blood and Tissue kits (Qiagen); midguts placed in micro-centrifuge tubes in 180 µl PBS were homogenised using disposable micro-tube pestles.

For each sample, 20 µl proteinase K and 200 µl buffer AL were added and mixed thoroughly by vortexing then incubated at 56ºC for 10 min. Ethanol (96-100%) was added to the sample with the amount of 200 µl and mixed thoroughly by vortexing. The mixture was pipetted into DNeasy Mini spin column placed in a 2 ml collection tube and the flow-through was discarded after centrifugation

at 8000 rpm for one minute. For washing the sample, 500 µl of washing buffer AW1 was added and centrifuged for one minute at 8000 rpm. Another washing step was done using 500 µl of the washing buffer AW2 and centrifuged at 14,000 rpm for three minutes. DNA was eluted by placing the DNeasy Mini spin column in a clean micro-centrifuge tube and adding 200 µl of elution buffer AE directly on the DNeasy membrane. After incubation at room temperature for one minute, the tubes were centrifuged at 8000 rpm for one minute.

7.3.1.5.2 Reaction conditions

Primers targeting the alpha-elongation factor specific for *Glossina* species, reported by Mathew (2007) were used for qPCR. The sequences of the primers are TseAF: 5'-CGG CTG GCA CGG TGA TAA CAT-3' and TseAR: 5'-GCG GGA GGG TGG CAA CATT-3', they amplify 121 bp amplicon.

The amplification mixture contained 1 µl DNA template, 300 nM of each primer, 5 µL SYBR Green I ready-made master mix supplied by Qiagen (containing HotStarTaq DNA Polymerase, Quantitect SYBR Green I PCR Buffer [Tris-Cl, KCl, $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, pH 8.7], 0.2 mM dNTP mix, SYBR Green I dye, ROX dye), 0.1 units AmpErase [Uracil N-glycosylase] (Applied Biosystems). Water was added to a final volume of 10 µl.

The reaction conditions used were 50ºC for two minutes to activate UNG, 95ºC for 15 min then 40 cycles at 95ºC for 45 sec, 57ºC for 45 sec and 72ºC for 45 sec, plate read for fluorescence acquisition was set up after the extension step following the first 15 cycles to get rid of fluorescence noise that occurs before the amplification of the product. A temperature gradient between 55ºC and 95ºC was run to obtain the dissociation curve. Amplicons were visualised using 1% agarose gel to confirm specificity of the reaction. The standards and samples were run in triplicates and expressed as a mean value, non-template controls were also used to check the presence of contamination.

7.3.2 Optimisation of fly age and trypanosome infective dose

Prior to application of the single and mixed infections for the qPCR and haemocytometer quantification, flies were infected with a wide range of parasites from one to 50,000 parasites per bloodmeal. This was done to optimise the infective dose that would result in an infection of 100% of the flies when GSH was used to increase the infection rate.

7.3.2.1 Tsetse fly origin and maintenance

Glossina morsitans morsitans (Westwood) were originally from the Langford colony established at Bristol from pupae collected from Zimbabwe in 1967; the colony has been at University of Edinburgh for 10 years. Tsetse flies were kept at $25^oC \pm 1^oC$, at 70% relative humidity. Pupae were collected from deposition trays and placed into an emergence cage. Once tsetse flies had emerged they were

chilled at 4°C for 10 min and at this time were separated into males and females, placed in cages and allowed to rest for 24 h before being infected.

Tsetse flies were fed through an artificial silicon membrane system (Mews, 1980) three to four times a week. Defibrinated blood was poured on to trays on heating blocks (37°C) and covered with a silicon membrane, caged tsetse flies were then placed onto the membrane covered with a dark cloth and allowed to feed for 10 min.

7.3.2.2 Infection of tsetse flies with procyclic forms

Tsetse flies had their wings clipped on emergence and were allowed to rest for 24 h before being infected with trypanosomes. Cultured procyclics were centrifuged at 2500 rpm for 5 min and the culture supernatant removed, trypanosomes were then resuspended in 5 ml of ovine blood at a concentration of 33 to 1.67×10^6 /ml which is equivalent to one to 50,000 parasites per blood meal, respectively.

Infective bloodmeals were supplemented with 15 mM GSH (MacLeod *et al.*, 2007) in order to increase the midgut infection rate. As the infection rate in this colony is low <20% it was decided for practical reasons that it would be better to increase the midgut infection rate. The infective bloodmeal was then placed on a heated tray and covered with a silicon membrane. Tsetse were then placed on the membrane and allowed to feed. Flies that did not feed were removed from the experiment.

Teneral male *G. m. morsitans* received an infective bloodmeal containing procyclic *T. b. brucei* isolate (BUT 135) and/or *T. congolense* Savannah isolate (Sikudo 124 isolated from a cow in Sikudo, Tororo district, Uganda, 1990) supplemented with 15 mM GSH.

7.3.2.3 Dissection of the flies

Tsetse flies were chilled at 4°C for 30 min then kept on ice until dissection. Flies were dissected in saline 15 days post-infection; midguts were homogenised in 100 µl PBS and then placed on to FTA®cards.

7.3.2.4 Preparation of DNA for PCR

Samples applied onto FTA®cards were processed by eluting DNA from 10 discs (0.2 mm) using Chelex®100 as previously described in Chapter IV (section, 4.3.2.2.3). The same eluate was used for the conventional PCR reactions on each sample.

7.3.2.5 Conventional PCR

The primer sequences, reaction conditions and the size of the amplified products of TBR-PCR and TCS-PCR are detailed in chapters IV and V, respectively.

7.3.3 Single and mixed infection experiment

7.3.3.1 Infection of tsetse flies with procyclic forms

For qPCR and haemocytometer quantification, flies were infected with $1.67x10^6$ parasites/ml blood (equivalent to 50,000 parasites/bloodmeal). Dissected midguts were placed in a micro-centrifuge tube containing 200 µl PBS for DNA extraction. Twenty microliters were taken out for counting trypanosomes using haemocytometer and the remaining 180 µl was used for DNA extraction from midguts for qPCR.

7.3.3.2 Dissection of the flies

Flies were dissected 15 days post-infection as previously described in section 7.3.2.3 of this chapter.

7.3.3.3 Preparation of DNA for qPCR

DNA from the dissected midguts was extracted as previously mentioned in section 7.3.1.5.1 of this chapter.

7.4 Statistical analysis

The qPCR amplification efficiency (E) determined by linear regression of the standard curve was calculated from the slope (s) of the standard curve using the equation: $E=10^{-1/s}$ -1 (Klein *et al.*, 1999). The acceptable efficiency of the qPCR assay should be between 90-110%.

Inter assay precision was calculated using the following formula (Murray *et al.*, 1993): Inter-assay precision= (Standard deviation of the mean Ct of the triplicates/ Grand mean Ct of the triplicates) x 100

Normality of the data distribution was tested using the *Anderson-Darling* normality test in Minitab version 15 (Minitab, Inc.). The data were considered not normally distributed when p-value was <0.05.

Correlation between haemocytometer and qPCR quantification and *Mann-Whitney U* test were calculated using Minitab version 15 (Minitab, Inc.). *Kruskal-Wallis* one way analysis of variance

(ANOVA) and *Dunn's* multiple comparison tests were calculated using GraphPad Prism, Inc., version 5.02.

7.5 Results

7.5.1 Quantitative PCR

Prior to the application of the single and mixed experimental infections of *G. m. morsitans*, different qPCR reactions were optimised and evaluated. The reactions include specific qPCR for *T. brucei* s.l., *T. congolense* Savannah and *Glossina* species.

7.5.1.1 Evaluation and optimisation of *T. brucei* s.l. qPCR

Two reactions for the quantification of *T. brucei* s.l. were used in the current study. The first reaction was the one previously reported by Becker *et al*. (2004) targeting a satellite DNA sequence. The second reaction targeting a single copy gene was optimised in the current study to get rid of the pitfalls encountered using the first reaction to be used in qPCR.

7.5.1.1.1 *T. brucei* s.l. qPCR using satellite DNA target

A qPCR for quantifying *T. brucei* s.l. DNA in human blood samples collected on FTA®cards has been reported by Becker *et al*. (2004). The authors re-designed the primers targeting the 177 bp repetitive DNA satellite sequence (Moser *et al.*, 1989a) to get rid of the tendency of the primers to form secondary structures.

The qPCR performance was tested using the primers on *T. b. brucei* extracted DNA with a concentration ranging from $4x10^6$ to 40 parasites/ml using Applied Biosystems kits. The log linear standard curve was obtained by plotting the Ct values of the assays versus the input parasite numbers obtained from five reactions (Figure 7.8).

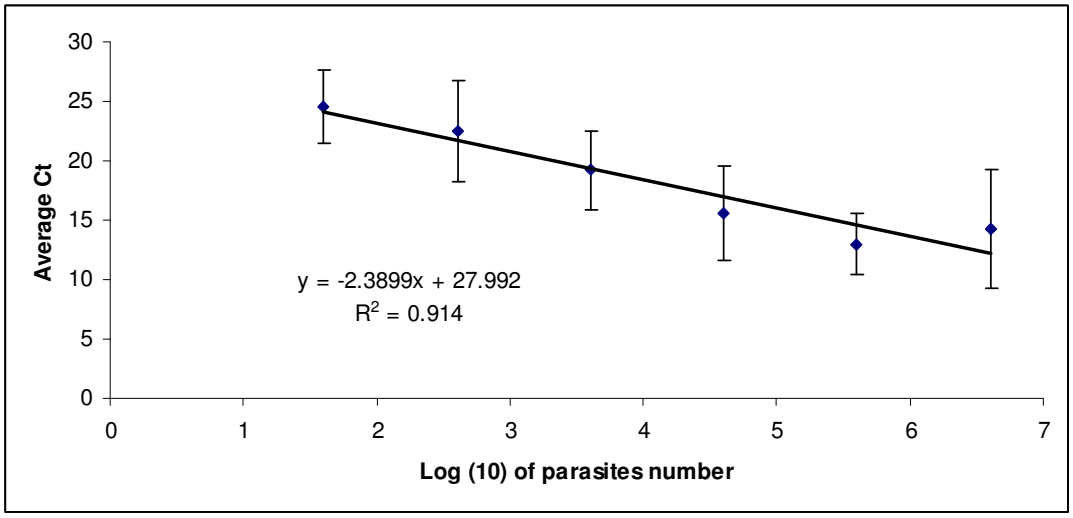

Figure 7.8: Average standard curve of cycle threshold (Ct) versus log (10) of *T. b. brucei* concentration/ml using AB kits. Data points were obtained by calculating the mean Ct across sample sets for each standard concentration from five reactions. Vertical bars represent standard deviations

Figure 7.8 shows that low Pearson correlation coefficient (R^2=0.914) was obtained and this indicates that the efficiency of amplification was not consistent at varying template concentrations. The reaction efficiency was calculated from the slope and it was found to be 162.1%.

The inter assay precision was calculated in five repeats of standards and found to be more than 10% (12.5-35.3%) which is higher than the acceptable range. This indicates lower reproducibility of the assay showing variation over the six orders of magnitude which lower the precision of the applied assay.

The specificity of the reaction was determined using different concentrations of other trypanosome DNA including *T. congolense* Savannah and *T. vivax*, and also using different concentrations of *Glossina* DNA. The results showed cross reactivity of the primers with *T. congolense* Savannah, *T. vivax* and *Glossina* genomic material especially after running the reaction more than 25 cycles. Most importantly, multiple bands were observed using the *T. b. brucei* DNA which makes this reaction not ideal for qPCR quantification.

In order to justify the use of another qPCR reaction targeting another DNA sequence in *T. brucei* s.l. genomic material, the satellite DNA primers were evaluated using other kits (BioRad and Qiagen). Figures 7.9 and 7.10 show the standard curves poltted from the Ct values of the assays versus the input parasite numbers obtained from nine reactions, each.

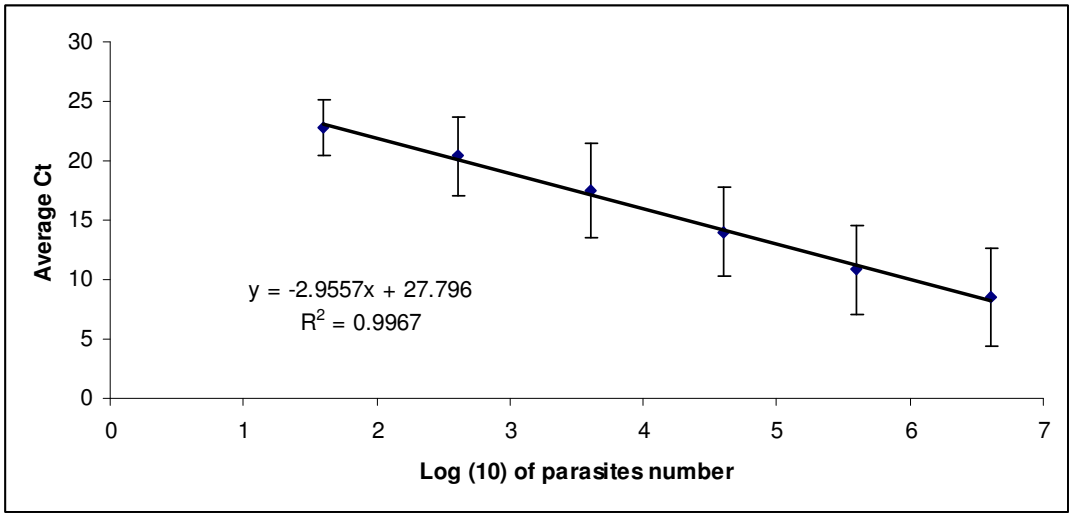

Figure 7.9: Average standard curve of cycle threshold (Ct) versus log (10) of *T. b. brucei* concentration/ml using BioRad kits. Data points were obtained by calculating the mean Ct across sample sets for each standard concentration from nine reactions. Vertical bars represent standard deviations

Figure 7.9 shows that a high Pearson correlation coefficient (R^2=0.996) was obtained and this indicates that the reaction was linear at varying template concentrations. However, the vertical bars showing standard deviations indicate variation between different runs of the standards. The reaction efficiency was calculated from the slope and it was found to be 117.9%.

The inter assay precision was calculated in nine repeats of standards and found to be more than 10% (10.5-48.8%) which is higher than the acceptable range. This indicates lower reproducibility of the assay showing variation over the six orders of magnitude which lowers the precision of the assay.

The specificity of the reaction was determined using different concentrations of other trypanosome DNA including *T. congolense* Savannah and *T. vivax*, and using also different concentrations of *Glossina* DNA. The results showed cross reactivity of the primers with *T. congolense* Savannah, *T. vivax* and *Glossina* genomic material especially after running the reaction more than 25 cycles. Moreover, multiple bands were observed using the *T. b. brucei* DNA which makes this reaction not ideal for qPCR quantification.

Figure 7.10 shows the standard curve plotted from the Ct values of the assays versus the input parasite numbers obtained from nine reactions using Qiagen kits.

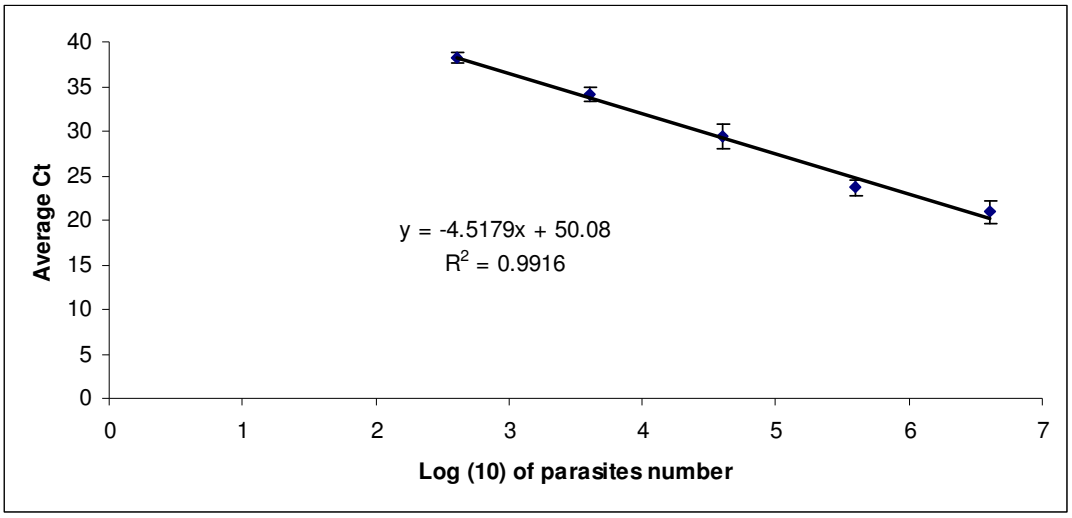

Figure 7.10: Average standard curve of cycle threshold (Ct) versus log (10) of *T. b. brucei* concentration/ml using Qiagen kits. Data points were obtained by calculating the mean Ct across sample sets for each standard concentration from nine reactions. Vertical bars represent standard deviations

The results show that a high Pearson correlation coefficient (R^2=0.991) was obtained and this indicates that the reaction was linear at varying template concentrations. The reaction efficiency was calculated from the slope and it was found to be 66.5%.

The inter assay precision was calculated in nine repeats of standards and found to be less than 10% (1.7-5.9%) which is within the acceptable range. This indicates the reproducibility of the assay showing minimal variation over the five orders of magnitude with high precision of the applied assay. However, the sensitivity of the reaction using Qiagen kits was 400 parasites/ml with more cycle numbers (40 cycles).

The specificity of the reaction was determined using different concentrations of other trypanosome DNA including *T. congolense* Savannah and *T. vivax*, and using also different concentrations of *Glossina* DNA. The results showed cross reactivity of the primers with *T. congolense* Savannah, *T. vivax* and *Glossina* genomic material especially after running the reaction more than 25 cycles. Most importantly, multiple bands were observed using the *T. b. brucei* DNA and we may conclude that this reaction is not ideal for qPCR quantification (Figure 7.11).

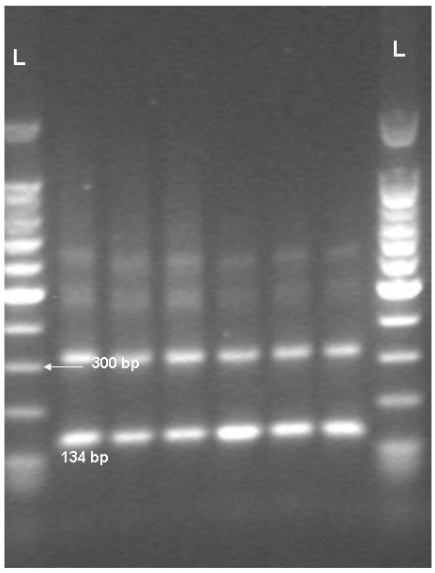

Figure 7.11: Multiple bands obtained by *T. brucei* s.l. qPCR using satellite DNA target. Amplified product size is 134 bp and double band at more than 300 bp with multiple band patterns are shown. L: 100 bp ladder

7.5.1.1.2 *T. brucei* s.l. qPCR using a single copy gene target

Another qPCR reaction specific for *T. brucei* s.l. was optimised in the current study for the quantification of *T. b. brucei* infections in midguts. The target in this reaction is the single copy PLC gene specific for *T. brucei* s.l. (Mensa-Wilmot *et al.*, 1990). Figure 7.12 shows the standard curve plotted from running the same standards for 18 reactions.

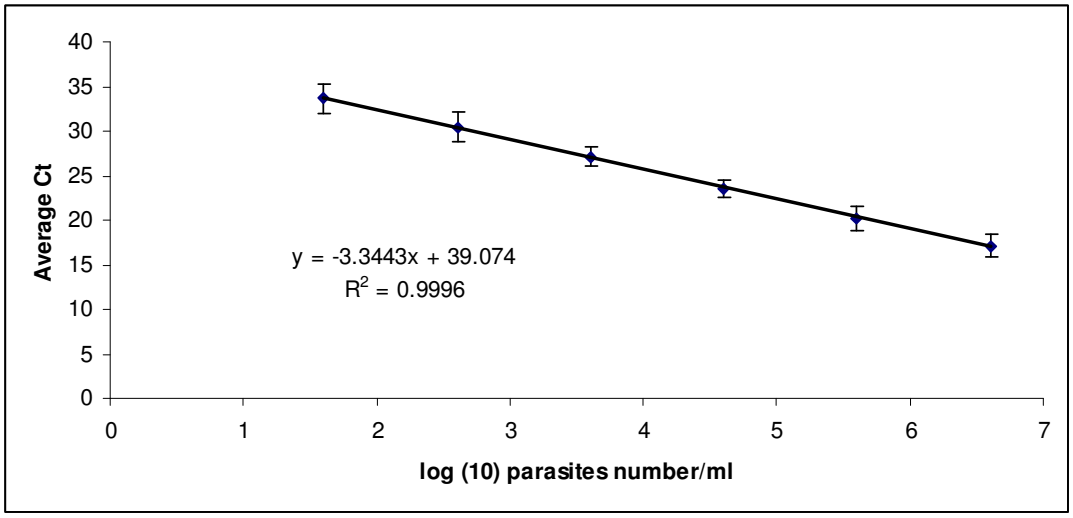

Figure 7.12: Average standard curve of cycle threshold (Ct) versus log (10) of *T. b. brucei* concentration/ml. Data points were obtained by calculating the mean Ct across sample sets for each standard concentration from 18 reactions. Vertical bars represent standard deviations

Figure 7.12 shows that a high Pearson correlation coefficient (R^2=0.9996) was obtained and this indicates a linear standard curve which implies that the efficiency of amplification was consistent at varying template concentrations. The efficiency was calculated from the slope and it was found to be 99.1%.

The inter assay precision was calculated in 18 repeats of standards and found to be less than 10% (4.2-7.6%) which is within the acceptable range. This indicates the reproducibility of the assay over six orders of magnitude showing minimal variation and high precision of the applied assay.

The sensitivity of the assay was evaluated using different amounts of *T. b. brucei* DNA by serial dilution of the starting amount over 10 orders of magnitude; however, R^2 value was too low indicating low linearity. By excluding the lower concentrations, the linearity was sufficient and the sensitivity of the reaction was 40 parasites/ml.

The specificity of the reaction was determined using different concentrations of other trypanosome DNA including *T. congolense* Savannah and *T. vivax*, and using also different concentrations of *Glossina* DNA. The results showed that the reaction was specific for *T. b. brucei* DNA and there was no amplification noticed using the other DNA material. We conclude that the primers used were suitable for use in qPCR due to obtaining one single band and lack of amplification of non target species.

7.5.1.2 Optimisation of *T. congolense* Savannah qPCR

Primers optimisation

Primers used for the conventional PCR in the amplification of a satellite DNA sequence in *T. congolense* Savannah (Masiga *et al.*, 1992; Moser *et al.*, 1989a) were re-designed in the current study by excluding one base from the reverse primer sequence for use in qPCR. The primer concentration was optimised using different combination of the two primer sets ranging from 50 nM to 300 nM, the primer pair that resulted in lower Ct value were chosen to be used for the qPCR. Table 7.1 shows the different Ct values obtained using different primer pair concentrations.

Table 7.1: Primer pair concentration optimisation for qPCR (red colored Ct value corresponds to the optimal primer pair concentration)

Reverse primer	**Forward primer**			
		50 nM	150 nM	300 nM
	50 nM	18	20.4	22
	150 nM	20.7	20.5	22.5
	300 nM	21	22	20

Table 7.1 shows Ct value obtained using different primer pair concentrations combination for optimisation. The results show that using 300 nM of both primres resulted in lower Ct value compared to the other combinations (20); therefore this concentration was used for the subsequent reactions in the current study.

Although 150 nM combination of primers resulted in lower Ct value than the chosen 300 nM concentration (18), it was not sufficient for amplification of lower parasite concentration and also resulted in background non-specific amplification.

Reaction conditions

BioRad kits were chosen to be used for qPCR of *T. congolense* Savannah genomic material because when compared to the other kits, lower Ct value was obtained with BioRad kits. The annealing temperature of the primers was evaluated with gradient temperature and temperature of 60ºC was found optimum. This temperature was sufficient for the annealing and extension steps as using a separate extension step at 72ºC resulted in non-specific amplification.

Figure 7.13 shows the standard curve plotted from running the same standards for 20 reactions.

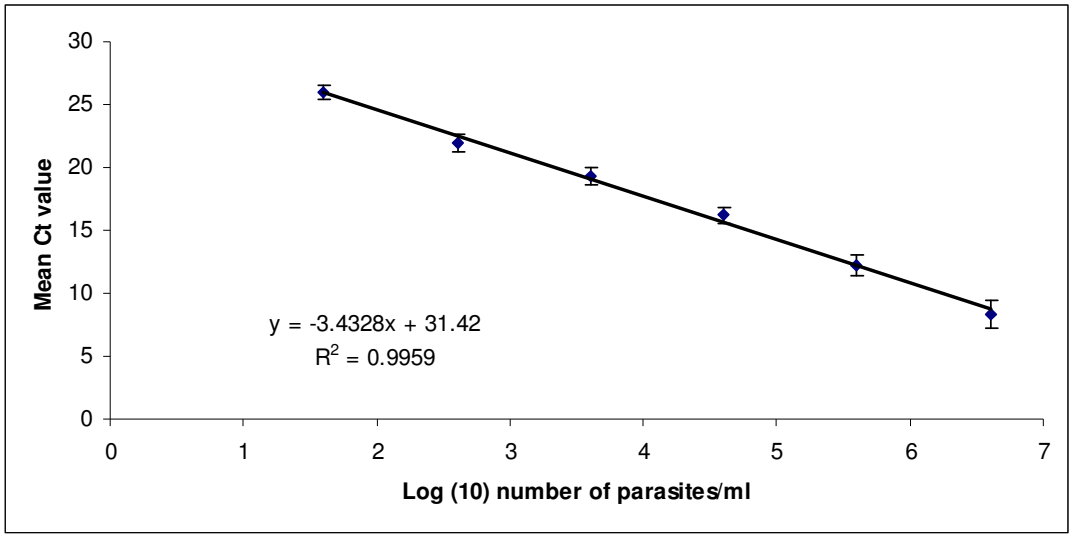

Figure 7.13: Average standard curve of cycle threshold (Ct) versus log (10) of *T. congolense* Savannah concentration/ml. Data points were obtained by calculating the mean Ct across sample sets for each standard concentration from 20 reactions. Vertical bars represent standard deviations

Figure 7.13 shows that a high Pearson correlation coefficient (R^2=0.9959) was obtained and this indicates a linear standard curve which implies that the efficiency of amplification was consistent at varying template concentrations. The efficiency was calculated from the slope and it was found to be 95.6%.

The inter assay precision was calculated in 20 repeats of standards and found to be less than 10% (2.2-6.9%) which is within the acceptable range. This indicates the reproducibility of the assay over six orders of magnitude showing minimal variation and high precision of the applied assay.

The sensitivity of the assay was evaluated using different amounts of *T. congolense* Savannah DNA by serial dilution of the starting amount over 10 orders of magnitude; however, R^2 value was too low indicating low linearity. By excluding the lower concentrations, the linearity was sufficient and the sensitivity of the reaction was found to be 40 parasites/ml.

The specificity of the reaction was determined using different concentrations of other trypanosome DNA including *T. b. brucei* and *T. vivax*, and using also different concentrations of *Glossina* DNA. The results showed that the reaction was specific for *T. congolense* Savannah DNA and there was no amplification noticed using the aforementioned DNA materials. The primers used were shown to be more suitable for use in qPCR due to obtaining one single band.

7.5.1.3 Optimisation of *Glossina* species qPCR

Glossina qPCR targets a two copy gene (alpha-elongation factor gene) in the tsetse genome (Degnan *et al.*, 2004). The reaction was reported by Mathew (2007), however, the primer concentration of 500 nM was shown to produce primer dimers and increased the background fluorescence. Therefore, the primer concentration was reduced to 300 nM which lowered the formation of primer dimers and removed background fluorescence.

Also, Mathew (2007) reported the use of Q-solution in the reaction mixture; this solution changes the melting behaviour of DNA and can be used for PCR systems that do not work well under standard conditions (Qiagen, 2008). However, this solution could cause reduced efficiency or failure of a previously successful amplification reaction. In order to evaluate the use of Q-solution in the current reaction, parallel reactions with and without Q-solution were performed on replicates of the same standard concentration containing 93.4 ng/µl (Table 7.2).

Table 7.2: Ct value and melting temperatures of amplified products with and without Q-solution

With Q-solution		**Without Q-solution**	
Ct value	Tm	Ct value	Tm
39.73	76	24.79	81
29.94	79	25.51	81
30.64	78	25.61	81
29.68	79	25.34	81
31.52	78	25.23	81
29.9	78	25.4	81

The results in Table 7.2 show that using Q-solution resulted in higher Ct values compared to the values obtained without using Q-solution. Moreover, Q-solution changed the melting behaviour of DNA giving multiple Tm of the same product; while without Q-solution, Tm was constant.

Figure 7.14 shows the standard curve plotted from running the same standards for 16 reactions using *Glossina* DNA standards.

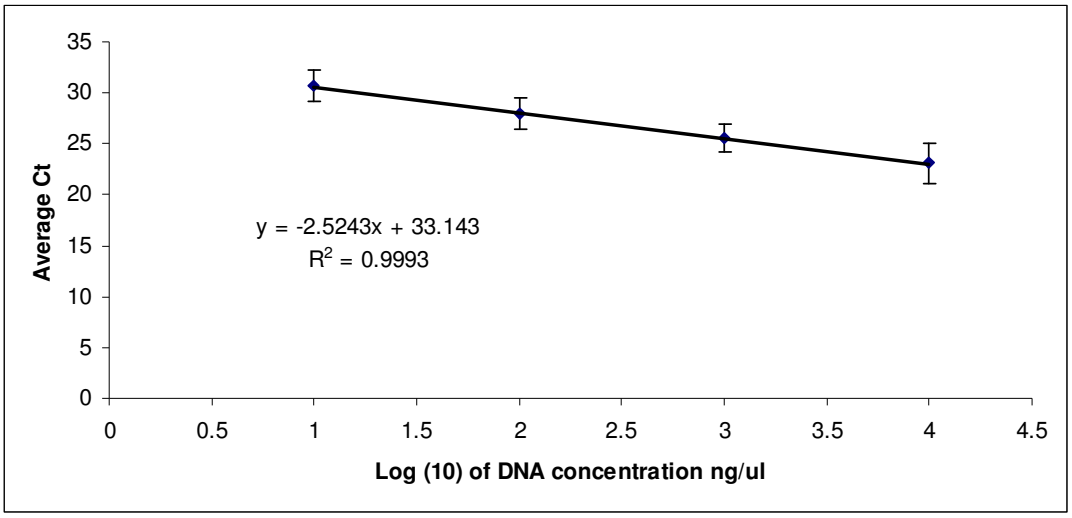

Figure 7.14: Average standard curve of cycle threshold (Ct) versus log (10) of *Glossina* DNA concentration (ng)/µl. Data points were obtained by calculating the mean Ct across sample sets for each standard concentration from 16 reactions. Vertical bars represent standard deviations

Figure 7.14 shows that a high Pearson correlation coefficient (R^2=0.9993) was obtained and this indicates a linear standard curve which implies that the efficiency of amplification was consistent at varying template concentrations. The efficiency was calculated from the slope and it was found to be 149%.

The inter assay precision was calculated in 16 repeats of standards and found to be less than 10% (5.1-8.2%) which is within the acceptable range. This indicates the reproducibility of the assay over four orders of magnitude showing minimal variation and high precision of the applied assay.

The sensitivity of the assay was evaluated using different amounts of *Glossina* DNA by serial dilution of the starting amount over 10 orders of magnitude; however, R^2 value was too low indicating low linearity. By excluding the lower six concentrations, the linearity was sufficient and the sensitivity of the reaction was 0.0934 ng/µl. This sensitivity was sufficient because it included the average amount of DNA calculated to be in a single fly (13.6 ng/µl).

The specificity of the reaction was determined using different concentrations of trypanosome DNA including *T. b. brucei, T. congolense* Savannah and *T. vivax*. The results showed that the reaction was specific for *Glossina* DNA and there was no amplification noticed using the aforementioned DNA materials. The primers used were shown to be more suitable for use in qPCR due to obtaining one single band (Figure 7.15).

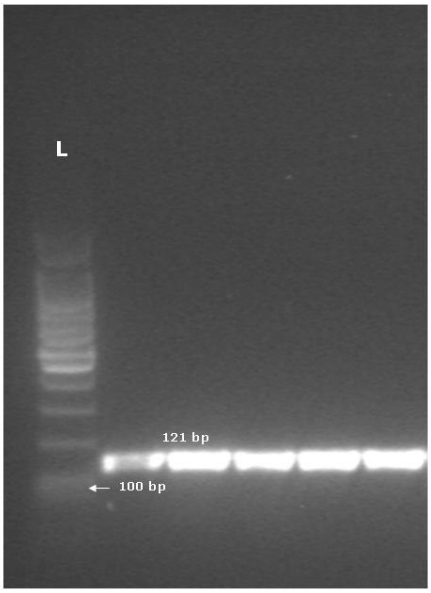

Figure 7.15: Example of amplified alpha-elongation factor gene in *Glossina* DNA using qPCR. The amplified size is 121 bp

7.5.1.4 Normalisation of the qPCR reactions using *Glossina* DNA as an internal control

Trypanosome DNA standards were spiked with a constant amount of *Glossina* DNA and the qPCR specific for *Glossina* DNA was run to quantify the amount of the spiked DNA. This aimed to evaluate if the fluorescence detected using *T. b. brucei* and *T. congolense* Savannah qPCR reactions was a result of the amplification of trypanosome DNA and not due to the presence of *Glossina* DNA in the spiked standards or the samples.

The NanoDrop quantification of the *Glossina* DNA extracted from non-infected flies revealed an average of 2600 ng/fly (13 ng/µl), while qPCR resulted in a nearly similar amount of *Glossina* DNA with an average of 2724 ng/fly (13.6 ng/µl).

An average Ct value of 28.7± 0.7 was obtained using all serial dilutions of *T. b. brucei* and *T. congolense* Savannah spiked with the constant amount of *Glossina* DNA. The calculated average Ct value was equivalent to 13.2 ng/µl of *Glossina* DNA.

7.5.2 Optimisation of fly age and trypanosome infective dose

7.5.2.1 Age of the flies

In the current study, the main aim was to evaluate qPCR for the quantification of *T. b. brucei* and *T. congolense* Savannah in single and mixed infections of tsetse. Therefore, fly age and the infective dose were optimised to obtain 100% infection of the flies. The age of the flies was categorised into three groups; group A were flies <48 hours at the day of infection; group B flies 24>48 hours at the day of infection and group C flies <24 hours old on the day of infection. The age groups were infected with a high infective dose (20,000 parasites/bloodmeal) and dissected 15 days post-infection. Table 7.3 shows the proportion of flies infected (detected by PCR) with *T. b. brucei* and *T. congolense* Savannah in the different age categories.

Table 7.3: Proportion of different age categories infected with trypanosomes (n=30)

Trypanosome species	Age category		
	Group A (<48 h)	Group B (24>48 h)	Group C (<24 h)
T. b. brucei	13.3% (4/30; 3.8-30.7)	33.3% (10/30; 17.3-52.8)	70% (21/30; 50.6-85.3)
T. congolense Savannah	0 (0/30; 0-9.5)	26.7% (8/30; 12.3-45.9)	70% (21/30; 50.6-85.3)

The results in Table 7.3 show the proportion of flies infected at different age categories with trypanosomes. The difference in the proportion of infected flies with *T. b. brucei* was statistically significant among the three age categories (χ_2^2=20.9, p<0.001). The 70% proportion of infection of the flies less than 24 hours (group C) at the time of infection was significantly higher than the 13.3% proportion of group A and 33.3% proportion of infection in group B, (χ_1^2=19.8, p<0.001) and (χ_1^2=8.1, p=0.004), respectively. However, there was no statistical significant difference in the proportion of flies infected with *T. b. brucei* between group A and B (χ_1^2=3.4, p=0.07).

Regarding *T. congolense* Savannah, the results in Table 7.3 show that the 70% proportion of flies under the age category C (<24 h) was significantly higher than 26.7% of flies under the age category B (24>48 h) (χ_1^2=11.3, p=0.001). However, none of the flies more than 48 hours old when infected had *T. congolense* Savannah infections.

7.5.2.2 Infective dose

The infective dose was optimised using flies of less than 24 hours age in order to obtain 100% infection rate. This was achieved by conducting a series of infections using infective doses ranging from one parasite to $5x10^4$ parasites per bloodmeal which is equivalent to 33 to $1.67x10^6$ /ml. The results of the different trials for infecting flies with *T. b. brucei* and *T. congolense* Savannah are summarised in Table 7.4.

Table 7.4: Proportion of flies infected with trypanosomes (n=30)

Trypanosome species	Infective dose (parasites/blood meal)							
	one	10	100	1000	5000	10,000	20,000	50,000
T. b. brucei	0	13.3% (4/30; 3.8-30.7)	23.3% (7/30; 9.9-42.3)	33.3% (10/30; 33.3%)	43.3% (13/30; 25.5-62.6)	56.7% (17/30; 37.4-74.5)	70% (21/30; 50.6-85.3)	100%
T. congolense Savannah	0	10% (3/30; 2.1-26.5)	16.7% (5/30; 5.6-34.7)	30% (9/30; 14.7-49.4)	36.7% (11/30; 19.9-56.1)	50% (15/30; 31.3-68.7)	66.7% (20/30; 47.2-82.7)	100%

The results in Table 7.4 show that 50,000 parasites/bloodmeal are sufficient for 100% infection rate of *T. b. brucei* and *T. congolense* Savannah in flies. Accordingly, in the single and mixed experimental infections using *T. b. brucei* and/or *T. congolense* Savannah, the flies used were of age category C (less than 24 hours) and the infective dose used was 50,000 parasites/bloodmeal.

7.5.3 Single and mixed trypanosome infections in tsetse

Five groups of *G. m. morsitans* were infected with *T. b. brucei* and *T. congolense* Savannah to evaluate qPCR in quantifying trypanosome infections of the flies when infected with single and mixed species.

7.5.3.1 Comparison of qPCR and haemocytometer quantification

Experimentally infected flies with single infection of *T. congolense* Savannah and *T. b. brucei* were dissected 15 days post-infection and the infection load was estimated using both haemocytometer and qPCR quantification.

The correlation between using haemocytometer and qPCR quantification of *T. b. brucei* and *T. congolense* Savannah in single infected groups was calculated. The results revealed a significant correlation ($p<0.001$) between the two methods in quantifiying *T. b. brucei* (Pearson correlation= 0.969), Figure 7.16 shows a scatterplot for the correlation between qPCR and haemocytometer quantification of *T. b. brucei*.

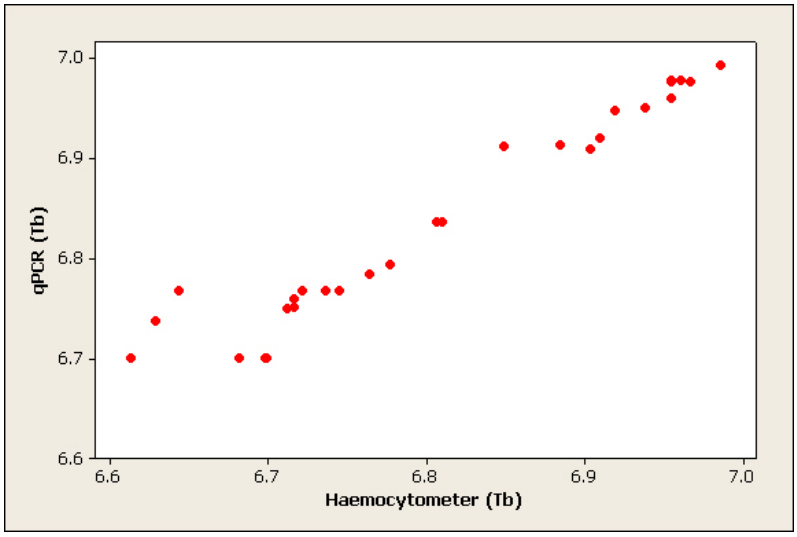

Figure 7.16: Scatterplot showing the correlation between qPCR and haemocytometer quantification of *T. b. brucei* (Tb) in midguts (numbers are presented on log 10 scale and each dot is the average of three replicates)

A significant correlation ($p<0.001$) was detected between the haemocytometer and qPCR quantification of *T. congolense* Savannah (Pearson correlation= 0.968), Figure 7.17 shows a scatterplot of the correlation between qPCR and haemocytometer quantification of *T. congolense* Savannah.

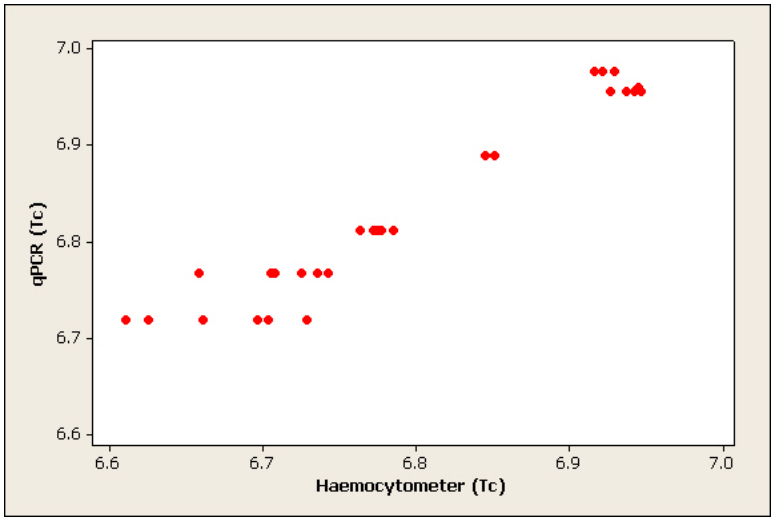

Figure 7.17: Scatterplot showing the correlation between qPCR and haemocytometer quantification of *T. congolense* Savannah (Tc) in midguts (numbers are presented on log 10 scale and each dot is the average of three replicates)

The detection limit of qPCR and haemocytometer to *T. b. brucei* and *T. congolense* Savannah was compared using *Mann-Whitney U* test which is used for non-parametric, independent samples and tests the null hypothesis that the two sample medians being compared are equal. Comparing the medians, rather than the means, of the samples is more accurate in this study as they better represent the centre of a sample-set that is not normally distributed and results are not influenced by outliers. Figure 7.18 shows a boxplot of the log (10) number of parasites/midgut quantified using qPCR and haemocytometer for the two trypanosome species.

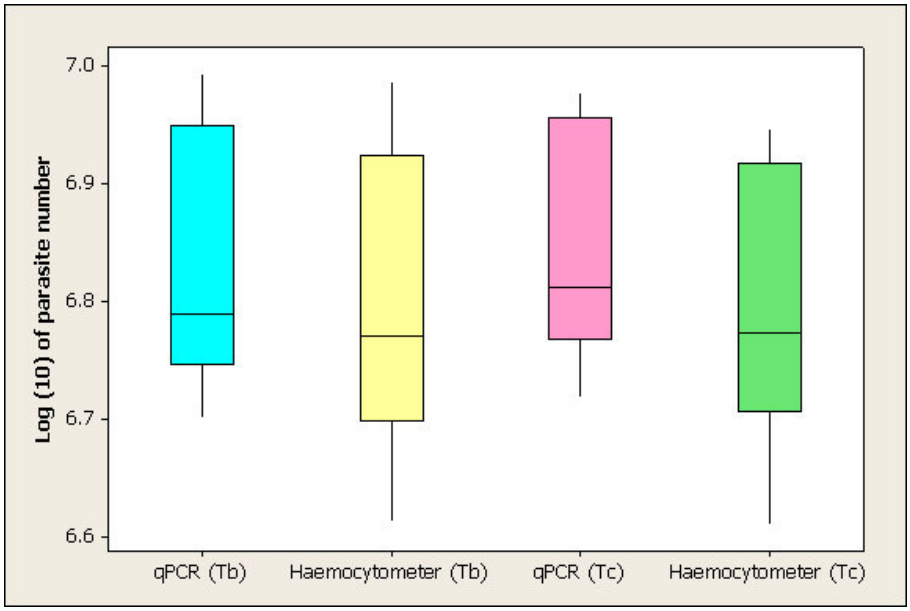

Figure 7.18: Boxplot showing the median log (10) number of parasites (Tb: *T. b. brucei*, Tc: *T. congolense* Savannah) quantified using qPCR and haemocytometer in the single infected groups (Numbers are presented as log 10 scale)

Figure 7.18 shows that the median log number of *T. b. brucei* quantified with qPCR was 6.79 ($6.2x10^6$) which is insignificantly higher than the median log number of parasites ($6.77=5.9x10^6$) obtained using haemocytometer method (p=0.18). Moreover, the median number of *T. congolense* Savannah obtained by qPCR ($6.81=6.5x10^6$) was not significantly higher than the median number ($6.77=5.9x10^6$) obtained with haemocytometer quantification (p=0.1).

7.5.3.2 Infection load of *Glossina* species groups with single and mixed trypanosome infections

7.5.3.2.1 Infection load of *Glossina* with *T. b. brucei* single and mixed infections

The infection load of *G. m. morsitans* with *T. b. brucei* when in single and mixed infection was compared in the four fly groups. The first group of flies were infected with a single infection of *T. b. brucei*; the second group of flies were infected simultaneously with *T. b. brucei*/*T. congolense* Savannah; the third group of flies were infected firstly with *T. b. brucei* and after two days another feed infected with *T. congolense* Savannah; finally, the fourth group of flies were infected firstly with *T. congolense* Savannah followed by *T. b. brucei* after two days. Figure 7.19 shows a boxplot of the *T. b. brucei* quantification in the four groups.

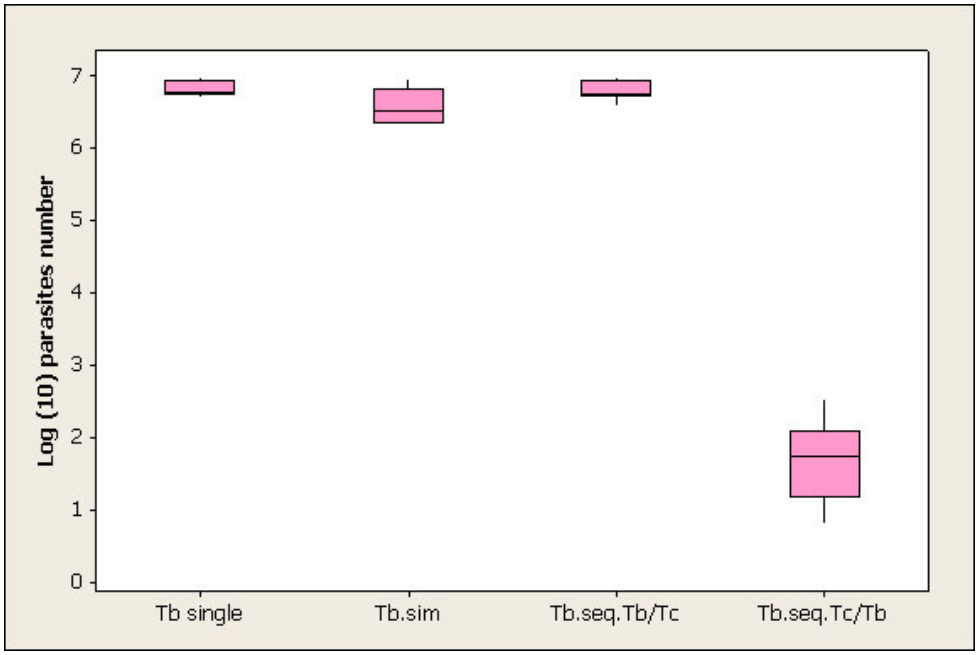

Figure 7.19: Boxplot for *T. b. brucei* quantification in the four groups (Tb: *T. b. brucei*; sim: simultaneous; Tc: *T. congolense* Savannah; seq: sequential, Numbers are presented as log 10 scale)

Figure 7.19 shows that the median number of *T. b. brucei* in the single infection group was 6.79 ($6.2x10^6$), while it was 6.51 ($3.3x10^6$), 6.77 ($5.9x10^6$), and 1.75 (6.3x10) in simultaneous *T. b. brucei*/*T. congolense* Savannah, sequential *T. b. brucei*/*T. congolense* Savannah and sequential *T. congolense* Savannah/*T. b. brucei* infection group, respectively. The infection rate in the four groups was 100% for both species.

The data were shown to be not normally distributed; therefore, the median of the four groups was compared using *Kruskal-Wallis U* one way ANOVA which is a non-parametric test. To test the difference in between the four groups, *Dunn's* test was used as a multiple comparison post test. The results show that there was a significant difference in the median of *T. b. brucei* number in the four groups (H_3=74.03, $p<0.001$).

There was no statistical significant difference between the median number of *T. b. brucei* when present in single infection, when fed simultaneously and when first fed in the sequential infection ($p>0.05$). However, the median number of *T. b. brucei* fed secondly in the sequential infection with *T. congolense* Savannah was significantly lower than the other three groups ($p<0.05$).

7.5.3.2.2 Infection load of *Glossina* with *T. congolense* Savannah single and mixed infections

The infection load of *G. m. morsitans* with *T. congolense* Savannah was compared in the four groups, the four groups were; flies with single infections, simultaneous infected flies with *T. b. brucei*/*T. congolense* Savannah, flies infected sequentially with *T. b. brucei* and after two days another feed infected with *T. congolense* Savannah, finally, flies infected sequentially with *T. congolense* Savannah followed by *T. b. brucei* after two days. None of the flies infected sequentially with *T. b. brucei* and after two days another feed infected with *T. congolense* Savannah had *T. congolense* Savannah infections. Figure 7.20 shows a boxplot for the *T. congolense* Savannah quantification in the four groups.

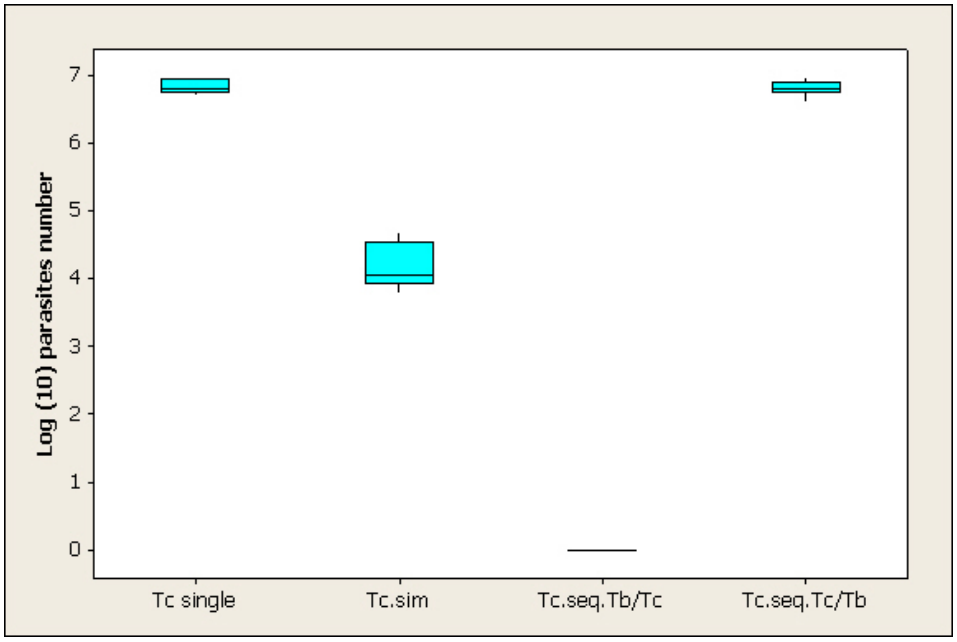

Figure 7.20: Boxplot for *T. congolense* Savannah quantification in the four groups (Tb: *T. b. brucei*; sim: simultaneous; Tc: *T. congolense* Savannah; seq: sequential, Numbers are presented as log 10 scale)

The median number of *T. congolense* Savannah in the single infection group was 6.81 ($6.5x10^6$); while it was 4.1 ($1.14x10^4$) and 6.81 ($6.5x10^6$) in the simultaneous *T. b. brucei/T. congolense* Savannah and sequential *T. congolense* Savannah/*T. b. brucei* infection group, respectively. The infection rate in the single, simultaneous and sequential *T. congolense* Savannah/*T. b. brucei* was 100% for both species, while, none of the flies infected with sequential *T. b. brucei/T. congolense* Savannah was infected with *T. congolense* Savannah.

The data were shown to be not normally distributed; therefore, the median of the four groups was compared using *Kruskal-Wallis U* one way ANOVA which is a non-parametric test. To test the difference in between the four groups, *Dunn*'s test was used as a multiple comparison post test. The results in Figure 7.20 shows that there was a significant difference in the median of *T. congolense* Savannah number in the four groups (H_3=102.6, $p<0.001$).

There was no statistical significant difference between the median number of *T. congolense* Savannah when present in single infection and when first fed in the sequential infection ($p>0.05$). However, the median number of *T. congolense* Savannah single infection and that first fed was significantly higher than when fed simultaneously($p<0.05$). Interestingly, none of the flies fed sequentially with *T. congolense* Savannah in the second feed were found infected with *T. congolense* Savannah.

7.6 Discussion

Quantitative PCR was used in the current study to quantify the number of trypanosomes in the midgut of infected flies with single and mixed infections. The aim of this work was to evaluate and investigate the use of qPCR in the determination of midgut populations of *T. b. brucei* and *T. congolense* Savannah in single and mixed infections and studying the relationship between the two parasites in the fly midgut. Prior to application of the single and mixed experimental infections, the qPCR reactions specific for *T. b. brucei*, *T. congolense* Savannah and *Glossina* species were optimised and evaluated.

7.6.1 Evaluation and optimisation of *T. brucei* s.l. qPCR

T. brucei s.l. qPCR using satellite DNA target

SYBR Green I is an intercalating dye that binds to any double stranded DNA. Quantitative PCR using SYBR Green I chemistry requires a target that should have less tendency of forming secondary structures (primer dimers and non-specific amplifications) which affect quantification because SYBR Green I does not differentiate between different double stranded DNA. Therefore, if a target that tends to form secondary structures is used, fluorescence intensity would be collected from all double stranded structures resulting in inaccurate quantification.

The qPCR reaction specific for *T. brucei* s.l. using the Becker's primers showed multiple bands after amplification as visualised by gel electrophoresis in the current study (Figure 7.11) even though the authors re-designed the primers to lower the tendency of forming secondary structures. This pitfall impaired the quantification accuracy due to increasing the intensity of fluorescence collected from the multiple DNA structures. The tendency of the target which is a satellite sequence in *T. brucei* s.l. genome to produce multiple bands is due to the high copy number of the sequence in the genome which reaches 10,000 copies (Moser *et al.*, 1989a). The ladder pattern was reported by Artama *et al.* (1992) who argued that it results from the primers being extended through multiple tandem copies of the short satellite sequence during each PCR cycle. This repetitive target is considerd ubiquitous for the parasites and is the most favoured for the conventional identification of *Trypanozoons*; however, for qPCR quantification this target is not ideal.

Becker *et al.* (2004) reported that re-designing the primers was able to reduce the tendency of the primers to form secondary structures. The results of the current study are not in agreement with Becker *et al.* (2004) although the authors used a concentration range (10 to 10^7) higher than the concentration used in the current study (40 to $4x10^6$). The possible explanation of this difference is that Becker *et al.* (2004) used bloodstream forms of the parasites diluted in human blood which has factors that interfere with the PCR reaction, whereas, in the current study, pure DNA from procyclic cultures was used.

Becker *et al*. (2004) recommended AB kits for their reaction and they obtained an efficiency of 86% using serial dilution of the bloodstream form in human blood. In the current study, the efficiency obtained using AB kits was 162.1% which is higher than the acceptable range of 90-110%. This difference in efficiency could be attributed to the use of pure DNA from the parasites without any factors interfering with the qPCR reaction. Before the optimisation of another qPCR reaction specific for *T. brucei* s.l., two other kits (BioRad and Qiagen) were used. The efficiencies of the reaction were 117.9% and 66.5% using BioRad kits and Qiagen kits, respectively. In both cases, the efficiency was out side the acceptable limit of 90-110%, meaning that the amount of the product is not doubled with each cycle; this could be attributed to the formation of multiple structures.

The specificity of the reaction was evaluated using different concentrations of *T. congolense* Savannah, *T. vivax* and *Glossina* DNA. The reaction showed cross reactivity with the aforementioned genomic materials when running more than 25 cycles and this resulted in multiple non-specific bands that were documented with melting curve analysis and gel visualisation. Similar results were previously reported by Almeida *et al*. (1998) who reported cross-reactivity of the primers tragetting the satellite sequence of *T. brucei* s.l. with *T. vivax* and *T. congolense* stocks they examined.

The sensitivity of the reaction in the current study was calculated to be 40 parasites/ml with 30 amplification cycles; however, Becker *et al*. (2004) reported a sensitivity of 1000 parasites/ml with 40 amplification cycles. This strengthens the argument that mammalian DNA has an inhibitory effect on PCR reaction, therefore lowering the sensitivity of the reaction.

In conclusion, it was decided to optimise a different reaction specific for *T. brucei* s.l. for qPCR quantification instead of the reaction targeting the satellite DNA sequence to avoid the drawbacks encountered with the reaction, especially the production of secondary structures.

T. brucei s.l. qPCR using a single copy gene target

The single copy PLC gene is specific for *T. brucei* s.l. (Mensa-Wilmot *et al.*, 1990). This target was used in the current study for a qPCR reaction to quantify *T. brucei* s.l. in standards and *G. m. morsitans* midguts. The reaction conditions were previously reported by Picozzi *et al*. (2008); the conditions were evaluated for qPCR and optimised in the current study using serial dilutions of *T. b. brucei* DNA.

The efficiency of the reaction was calculated to be 99.1% which indicated that the amount of product doubled accurately with each cycle, this efficiency is within the acceptable 90-110% PCR efficiency. The inter assay precision also showed a minimal variation and high precision of the applied assay which indicated the reproducibility of the assay over the six orders of magnitude tested.

The sensitivity of the reaction was 40 parasites/ml and the reaction was specific for *T. b. brucei* with no production of secondary structures, which is ideal for qPCR.

7.6.2 Optimisation of *T. congolense* Savannah qPCR

In the current study, primers specific for *T. congolense* Savannah targeting a satellite DNA sequence of 5400 copies in the genome (Masiga *et al.*, 1992; Moser *et al.*, 1989a) were used for qPCR. The primers were re-designed to match the criteria of qPCR primers, where theoretical melting temperature of the two primers should be within 2ºC of each other. Therefore, one base was removed from the reverse primer sequence to obtain 2ºC difference. A concentration of 300 nM was chosen for both primers; this resulted in lower Ct value.

The reaction efficiency was 95.6% which shows sufficient doubling of the product amount with each cycle. The inter assay precision showed minimal variation indicating the reproducibility of the assay over six orders of magnitude.

The reaction sensitivity was 40 parasites/ml and the reaction was specific for *T. congolense* Savannah with no tendency of forming secondary structures even though it occurs at a high copy number (5400). There was no cross reaction of the primers with *T. brucei* s.l. and *T. vivax* DNA although the presence of similarity in the sequence of satellite DNA of *T. congolense* Savannah compared with *T. brucei* s.l. and *T. vivax* (44% and 37%, respectively), this did not affect the sensitivity of the primers to detect the target sequence (Masiga *et al*., 1992). This was studied by Gibson *et al*. (1987) who introduced the mini-chromosomes from *T. congolense* Savannah into *T. b. brucei* using electroporation. They were able to detect both satellite sequences indicating that these sequences are ideal for use in distinguishing trypanosome species.

7.6.3 Optimisation of *Glossina* species qPCR

A quantitative PCR reaction targeting the two copy alpha-elongation factor gene specific for *Glossina* DNA was used in the current study. This reaction was previously reported by Mathew (2007), however the primer concentration and reaction conditions were re-optimised to eliminate primer dimers and non-specific background fluorescence.

The reaction efficiency was calculated to be 149% which is more than the acceptable range of the efficiency meaning that the amount of product is not efficiently doubled with each cycle. Despite this finding, the reaction was used to confirm the presence of the *Glossina* DNA in constant amount in the standards spiked with *Glossina* DNA and in the experimentally infected flies.

The inter assay precision was minimal indicating the reproducibility of the assay over the four orders of magnitude. The sensitivity of the assay was 0.0934 ng/µl and the reaction was specific for *G. m. morsitans* with no cross reactivity with trypanosome DNA and with the production of a single amplicon which is ideal for qPCR.

7.6.4 Normalisation of the qPCR reactions using *Glossina* DNA as an internal control

Standards of *T. b. brucei* and *T. congolense* Savannah were spiked with a constant amount *of G. m. morsitans* DNA which is equivalent to the amount present in a single fly. This was done to mimic normal conditions and to ensure that the increase in fluorescence intensity was due to the increase in the number of parasites not due to the presence of the *Glossina* DNA. This was confirmed by obtaining the same average of *Glossina* DNA amount (13.2 ng/µl) from the different standard concentrations spiked with the same amount of *Glossina* DNA.

7.6.5 Optimisation of fly age and trypanosome infective dose

The age of *G. m. morsitans* and the infective dose of trypanosomes were optimised to obtain 100% infection rate in the flies; as the main aim of the study was to evaluate qPCR as a tool to measure trypanosome populations in the tsetse fly and to study the relationship between *T. b. brucei* and *T. congolense* Savannah when co-existing in midguts. Transmission of trypanosomes between hosts is entirely dependant on tsetse flies, but the flies are not easily infected due to the defence system of the tsetse against trypanosome invasion (Roditi and Lehane, 2008; Welburn and Maudlin, 1999). Tsetse were found to have midgut lectin activity that played a role in inhibiting trypanosome infections, feeding lectin inhibitory sugars (glucosamine) to flies along with the infective dose has shown to result in 100% infection rates. In the current study, GSH was used to increase the infection rate with an infective dose of 50,000 parasites/bloodmeal to obtain 100% infection rate to a level at which the relationship between the two parasites would be apparent. Glutathione is an antioxidant which protects trypanosomes from death induced in the fly midgut by reactive oxygen species (MacLeod *et al.*, 2007).

Fly age was known to be the most significant factor by far in *T. brucei* s.l. and *T. congolense* infections (Welburn and Maudlin, 1999). Several laboratory studies have reported that rates of infection decline in older flies (Jordan, 1978; Moloo and Shaw, 1989) and several studies have suggested that teneral flies are most susceptible to infection (Welburn and Maudlin, 1992). Otieno *et al.* (1983) found that flies fed on an infective bloodmeal less than 8 h after emergence produced higher levels of midgut infections than flies fed 12-24 h after emergence. This was attributed to the incomplete peritrophic membrane (PM) that makes younger flies easier to infect than older flies which have a fully formed PM. However, the addition of glucosamine to the infective feed of both teneral and non-teneral flies raised midgut infections to similar levels, suggesting that a fully formed PM is not a barrier to infection (Welburn and Maudlin, 1992). The addition of GSH to the blood meal for the three age category in the current study increased the infection rate of the flies aged <24 h with *T. b. brucei* and *T. congolense* Savannah. Flies with age less than 24 h and an infective dose of 50,000

parasites/bloodmeal were therefore chosen for the subsequent experiments to obtain a high midgut infection rate.

7.6.6 Single and mixed infection experiment of the flies

G. m. morsitans were infected with *T. b. brucei* and *T. congolense* Savannah using single and mixed infections. The infected flies were dissected 15 days post infection and the number of parasites in midguts was quantified using haemocytometer and qPCR in the single infected flies. However, only qPCR was used to estimate the number of parasites in midguts infected with mixed infections of the two species due to inability to differentiate between the two species when they co-exist together.

7.6.6.1 Comparison of qPCR and haemocytometer quantification

Quantification of *T. b. brucei* and *T. congolense* Savannah in the midguts of *G. m. morsitans* with single infections was estimated using haemocytometer and qPCR. The results showed that the median number of *T. b. brucei* and *T. congolense* Savannah quantified with qPCR was insignificantly higher than the median number obtained using haemocytometer. Moreover, the significant correlation between the two methods suggested that either haemocytometer or qPCR can be used for the quantification of trypanosomes and both are comparable. However, qPCR was chosen in the current study because the sensitivity of the haemocytometer quantification was $2.5x10^4$ parasites/ml compared to 40 parasites/ml for qPCR. Moreover, there was difficulty in differentiation between the two parasites in the mixed infected groups due to lack of experience in this field.

Quantification of *Leishmania* parasites in sand flies using haemocytometer and qPCR was reported by Ranasinghe *et al*. (2008). The results showed that qPCR absolute quantification was three times higher than haemocytometer quantification. These results are not enough to accurately compare the two methods because the authors applied the quantification on only six flies.

7.6.6.2 Trypanosome load of *Glossina* with single and mixed trypanosome species infections

Experimental infection of different *Glossina* species with single and mixed trypanosome species has been investigated by a number of authors in order to understand the relation between the different trypanosomes, to understand the cycle of transmission inside the vector and to estimate the burden of tsetse flies in the epidemiology of trypansosomiasis. However, none of the reported studies quantified the number of parasites in the fly midgut.

In the current study, the relationship between *T. b. brucei* and *T. congolense* Savannah was investigated by conducting single and mixed infections of *G. m. morsitans* and quantification of the

parasite number using qPCR. Figure 7.21 illustrates the median log number of *T. b. brucei* and *T. congolense* Savannah in single and mixed infection groups.

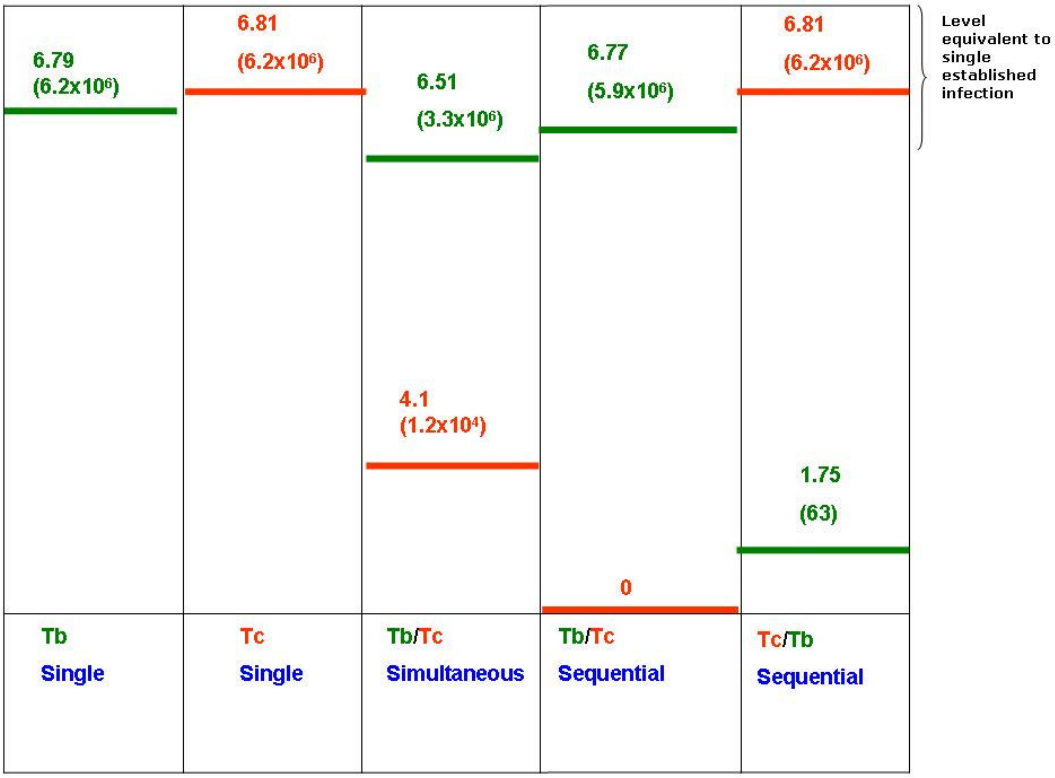

Figure 7.21: Illustration of the median log number (actual number between brackets) of *T. b. brucei* (green) and *T. congolense* Savannah (red) in single and mixed infection groups of *G. m. morsitans* (Tc: *T. congolense* Savanna, Tb: *T. b. brucei*)

The results in Figure 7.21 show that when *T. b. brucei* or *T. congolense* Savannah were fed to the flies once or in the first meal for the sequential mixed infection, the log number of parasites was consistently around 6.77 and 6.81. This could be attributed to the ability of the parasite to establish infection in the fly midgut when present in the first meal. This was in agreement with expectations from previous work that insects taking their first meal were much more easily infected by trypanosomes (reviewed in Welburn and Maudlin, 1999).

Looking at the log numbers in the mixed infection groups, the log number of *T. b. brucei* was significantly higher than *T. congolense* Savannah when fed to the flies in the same blood meal and all the flies had mixed infection of the two species. When *T. b. brucei* was fed to the flies in the first meal and *T. congolense* Savannah in the second sequential meal, there were no *T. congolense* Savannah infections detected at all. This suggests that the presence of *T. b. brucei* may compromise the ability of *T. congolense* Savannah to establish within the tsetse midgut.

The inability of *T. congolense* Savannah to establish an infection when fed after *T. b. brucei* could be down to several reasons. It is thought that trypanosomes control their numbers in the tsetse midgut through quorum sensing (Welburn and Maudlin, 1997). This could be achieved by the secretion of a factor which regulates trypanosome numbers (recently reviewed by Welburn *et al*., 2006). So it is possible that *T. b. brucei* secretes a molecule that will have a negative effect on the establishment of *T. congolense* Savannah. As *T. b. brucei* has several days head start under these conditions, its numbers may have reached a quantity to prevent the establishment of *T. congolense* Savannah. However, when the two species were fed simultaneously it may be that not enough *T. b. brucei* are present to affect the *T. congolense* Savannah parasites which still manage to establish, albeit several orders of magnitude smaller than when *T. congolense* Savannah was fed singularly. Following on from this, as *T. b. brucei* can establish a small population when fed after *T. congolense* Savannah, this species may be less prone to inhibition by the presence of other trypanosome species or simply better at establishing an infection. This is also suggested by the results of the simultaneous infection where *T. b. brucei* managed to establish at higher numbers than *T. congolense* Savannah.

Alternatively, it could be that a fly derived a factor that is responsible for the knock down of trypanosome numbers, with *T. congolense* Savannah being more susceptible than *T. b. brucei*. One thing to note is that further experiments will have to take place using different strains of *T. b. brucei* and *T. congolense* Savannah as the effect above could be a strain difference rather than a species related difference.

Gibson and Ferris (1992) designed an experiment to infect *G. m. morsitans* with simultaneous and sequential infection using *T. b. rhodesiense* and *T. congolense* Savannah. The flies were dissected 20 days post-infection and examined by DNA hybridisation probes specific for the two species. The results showed that 33.3% of the flies infected simultaneously with the two species had mixed infections. However, flies with sequential infection with *T. b. rhodesiense*/*T. congolense* Savannah, 54.3% of the flies had single infection with *T. b. rhodesiense* and 1.1% of the flies had single *T. congolense* Savannah and mixed infection of the two species, each. In case of sequential *T. congolense* Savannah/*T. b. rhodesiense*, 34.4% of the flies acquired single *T. congolense* Savannah infections, while, 1.04% had single *T. b. rhodesiense* and mixed infection of the two species, each. The authors concluded that it is possible to super-infect laboratory tsetse flies with *T. b. rhodesiense* and *T. congolense* Savannah by sequential feeding to mimic field conditions where flies have unrestricted freedom to feed on infected hosts suggesting that mixed infections can be acquired outside the narrow window of the first feed.

In the current work, GSH was used to boost the number of midgut infections, which would explain why an increased number of flies had midgut infections compared to the work of Gibson and Ferris (1992). This also explains why, in the current work, 100% of flies infected with *T. congolense* Savannah followed by *T. b. brucei* had infections of both trypanosome species, although the advantage of being fed first is clearly shown by the reduction in the population of *T. b. brucei* within

the fly midgut. However, similar effects were evident when *T. b. brucei* was fed first followed by *T. congolense* Savannah with the flies showing only *T. b. brucei* infections in the current work; by contrast only 1% of flies picked up a *T. congolense* Savannah infection in the work of Gibson and Ferris (1992).

The hypothesis of competition between trypanosome species was previously investigated by Seed (1978) who reported evidence of competition between two clones of *T. b. gambiense* when inoculated simultaneously into rats. One clone outgrew the other to an extent disproportionate to the difference in the replication rates of the two clones when inoculated on their own. Moreover, Jamonneau *et al.* (2004) inoculated *T. b. brucei*/*T. congolense* mixed infection into mice and found that *T. b. brucei* always out competed *T. congolense* which systematically disappeared.

Reifenberg *et al.* (1997b) simultaneously infected *G. m. morsitans* with *T. congolense* Savannah and *T. congolense* Forest and noted the exclusion of *T. congolense* Forest from the infection. This might indicate competition which could occur in the vector gut between the two species simultaneously ingested by the flies.

The development of *T. b. brucei* in *G. m. morsitans* midgut did not seem to be affected by the presence of *T. congolense* in the study of Van den Bossche *et al.* (2004a,b). This is in agreement with the current study where *T. b. brucei* developed in flies infected with *T. congolense* Savannah in the first meal although the median number of *T. b. brucei* was significantly lower than the median log number of *T. congolense* Savannah ($p<0.05$). In contrast, Kubi *et al.* (2005) suggested that previous exposure of tsetse flies to an infected blood meal or the presence of trypanosome has no effect on a subsequent secondary infection.

In conclusion, the current study suggests the possibility that a form of competition exists between *T. b. brucei* and *T. congolense* Savannah when they co-exist in the fly midgut. However, more research is required to determine the nature of such type of competition between the two species.

7.6.7 Applications

Investigation of tsetse infection load with trypanosomes and the relationship between the different species was previously done by haemocytometer counting, microscopy or conventional PCR. These methods were unable to accurately quantify the infection load of the flies or to estimate parasite numbers when they co-exist in the fly midgut. However, quantification of tsetse infection load with trypanosomes was reported by Peacock *et al.* (2007) using fluorescent markers of two *T. b. brucei* strains for studying the dynamics of infection and competition between the two strains. Although fluorescent trypanosomes may have some advantages over qPCR such as direct counting of trypanosomes, qPCR can be used on wild flies, which is obviously not the case with fluorescent trypanosomes.

The current study evaluated the use of qPCR to quantify the infection load of tsetse flies with *T. b. brucei* and *T. congolense* Savannah and to study the possibility of competition between the two species. The protocol could be used in the same manner to study the infection load of wild flies with different trypanosome species.

8 Chapter eight

General discussion

This thesis has concentrated on the molecular diagnosis of trypanosome infections obtained from a number of sources including cattle, wildlife and tsetse. The first two chapters formed literature reviews focussing on the general tools available for diagnosis of infections (Chapter I) using three examples of blood borne infectious parasites (*Plasmodium*, *Leishmania* and *Trypanosoma cruzi*) while Chapter II focussed on African trypanosomes as a model for blood borne parasites. The next five chapters formed the experimental work of this thesis and focussed on the following areas; evaluation of different sample preparations for trypanosome diagnosis, processing of samples from FTA®cards before PCR, evaluation of different PCR reactions in use for trypanosome identification, follow up of mass treatment campaign in Uganda and finally the application of qPCR for studying mixed infections in tsetse flies.

8.1 Advantages of molecular diagnosis

Whereas traditional in field diagnosis has relied on microscopy, evidence suggests that these techniques show low sensitivity, while serological tests suffer from the lack of separation of past and present infections. Molecular diagnosis through the use of PCR also allows infections to be characterised to the species/sub-species level. This is of great importance in differentiating the morphologically indistinct sub-species of *T. brucei* s.l., as *T. b. rhodesiense* has a zoonotic reservoir. In Uganda, domestic cattle play the role of the reservoir and due to the closeness between humans and cattle they provide an ideal stepping stone for infection of humans. Therefore, by removing the reservoir, the chance of human infection decreases. The advantages of molecular techniques in mapping an outbreak of Rhodesian sleeping sickness were shown by Fevre *et al*. (2001) who clearly demonstrated the importance of a cattle market at Brookes corner in Uganda as the focal point of the outbreak. Further to this, molecular characterisation of trypanosome infections has been an important part of several programmes to reduce the trypanosome reservoirs in cattle by using trypanocides. This has included the FITCA (Farming In Tsetse Controlled Areas) programme which finished in 2007 (Fyfe, 2007) and SOS (Stamp Out Sleeping sickness) programme which is still ongoing (Chapter VI).

8.2 Possible shortcomings of FTA®cards

In the first experimental chapter, it became clear that FTA®cards did not show similar levels of sensitivity when compared with in field DNA extraction. In the current work, preparations including venous blood, buffy coat and DNA applied on FTA®cards and extracted DNA solutions from whole blood and buffy coat were compared. The sensitivities of the different sample preparations were compared with a gold standard which was defined as positive PCR result from amplifying trypanosome DNA from FTA®cards and/or positive PCR result from amplifying trypanosome genomic material in DNA extract. The sensitivity of amplifying trypanosome DNA from DNA solutions was significantly higher than the amplification of DNA from FTA®cards even when examining multiple FTA discs. This was expected due to the uneven distribution of the parasite DNA

when in contact with the matrix after cell lysis. Therefore, it was decided to focus on methods which could improve the homogeneity of the blood samples which formed the basis of Chapter IV.

8.3 Processing of samples before PCR

The introduction of FTA®cards has greatly eased the transport of samples from where they are collected to laboratories where they can be analysed. However, although FTA®cards claim to produce a homogenous sample this is not always the case and success of the PCR can be determined by where the punch comes from on the card (Cox, 2007). Therefore, the current work investigated several methods which could increase the sensitivity of the reaction. In the laboratory, trypanosome samples from procyclic cultures and from blood samples collected from infected mice were lysed with various liquids before application on to FTA®cards. Lysis of infected blood promotes the release of the DNA into a more homogenous solution consisting of genetic material from both the host as well as any infectious agents. The probability of detecting the parasitic DNA in the examined material is therefore increased with a more even distribution of the genomic material throughout the solution. As the results of the experimental trials were promising, work was then moved into the field and two studies were carried out using water as the lysing agent.

A preliminary pilot study was conducted in field to obtain initial information about the logistics of lysis in the field; therefore, opportunistic samples were collected from 80 cattle in Uganda, in July, 2006. From these cattle, whole and lysed blood samples were collected and 10 discs were examined separately from each preparation by PCR. The results of screening the discs showed that increasing the number of the examined discs containing lysed blood samples to 10 discs resulted in 100% sensitivity compared with the gold standard (defined as a positive PCR result from amplifying trypanosome DNA from whole blood and/or lysed blood applied on FTA®cards). The increase in the sensitivity would suggest that although the distribution is improved by pre-lysis, it should not be assumed to be a homogenous distribution; by increasing the number of screened discs it is possible to improve the detection of trypanosome DNA. However, this level of sensitivity required the examination of 9-10 discs using a separate PCR reaction on each disc which is time consuming and expensive. Therefore, a larger field lysis study was conducted for further evaluation of the approach.

A large scale field study was conducted using a larger sample number (n=300) and three types of sample preparations, included whole blood on FTA®cards, lysed blood on FTA®cards and *in situ* DNA extraction. Examining 10 separate discs from FTA®card applied samples; the sensitivity of detecting positive discs containing lysed blood doubled the sensitivity of the screening protocol when compared to screening the same number of the discs containing whole blood. However, this improvement required the screening of at least 10 discs using separate PCR reactions. Therefore, to improve the detection of trypanosomes using only 10 discs with one PCR reaction for each species; DNA was eluted from FTA®card containing whole blood and lysed blood using 5% Chelex®100 resin aqueous suspension (Becker *et al.*, 2004). Chelex®100 is a chelating resin that has a high affinity for

polyvalent metal ions, moreover, the alkalinity (pH 10-11) and the exposure to temperature result in the disruption of the cell membrane and denaturation of DNA into its component single strands facilitating PCR amplification (Sweet *et al.*, 1996; Walsh *et al.*, 1991). The results showed that the sensitivity of identifying trypanosome DNA from whole blood and lysed blood eluate was 56.4% and 73.3%, respectively, compared to the gold standard (defined as PCR positive results from amplifying trypanosome DNA from samples applied to FTA®cards and/or positive PCR results from amplifying trypanosome genomic material in DNA extract).

The insignificant difference between using DNA eluted from whole blood, lysed blood FTA®card discs and DNA extracted using kits approves the use of any of the aforementioned preparations for obtaining trypanosome genomic materials. The choice should be based on economic and time factors; in field DNA extraction using kits is both expensive and time consuming. Pre-application lysis of infected blood products can help create a more homogenous distribution. It is possible to elute DNA from FTA®cards; this approach overcomes the uneven distribution of materials across the matrix. Therefore the choice between these two approaches could be based on the cost, for eluted whole blood sample the cost is £ 2.70 and for eluted lysed blood sample the cost is £ 3.20.

As the elution of DNA from FTA®cards showed no difference in sensitivity when compared with in field DNA extraction, this allowed the direct comparison of the ITS primer set with the species-specific primer sets. This would not have been possible using individual discs from FTA®cards as the results would not be directly comparable as the DNA content of the cards would have been different. This work was completed in Chapter V.

8.4 ITS-PCR versus species-specific PCR reactions

There are various primer sets available to identify trypanosome infections, however, screening each sample with up to five PCR reactions to identify to the sub-species level is time consuming and expensive. When this current work commenced, Cox *et al.* (2005) had recently published a set of primers which could discriminate trypanosomes to the species level (*T. brucei* s.l., *T. vivax*, *T. simiae* and *T. theileri*) or to the sub-species level (*T. congolense* Savannah, Forest and Kilifi). This of course has many advantages over single species PCR particularly in the fact that "one reaction fits all". Evaluation of the pan trypanosome ITS-PCR for the diagnosis of different trypanosome species compared to the species-specific PCR reactions was addressed in the current study. The comparison was based on the use of the same eluate to conduct the four PCR reactions; therefore the results would be comparable because the template is taken from the same eluate of the same discs from the card matrix.

The results documented the significant lower sensitivity of ITS-PCR in the diagnosis of *T. brucei* s.l. than the specific reaction targeting the high copy satellite DNA sequence. Firstly, this was thought to be attributed to the difference in the copy number of the ITS-PCR and TBR-PCR targets. This was

found not to be true as using a PCR reaction (PLC) that targets a single copy gene in *T. brucei* s.l. genome resulted in a significant higher sensitivity compared to ITS-PCR. Generally, during the current work it was found that ITS-PCR lacked sensitivity when compared to the single species reactions, this was independent of trypanosome species as the same trend was seen with *T. congolense* and *T. vivax*.

Due to the low sensitivity of the ITS primers set developed by Cox *et al*. (2005) it was decided to use the species-specific primers in conjunction with elution of DNA with Chelex®100 in Chapter VI to characterise the infection rate of trypanosomes in cattle following trypanocide treatment.

8.5 Stamp Out Sleeping sickness mass treatment campaign

The Chelex®100 and species-specific PCR reactions were used to evaluate and follow up the impact of a treatment programme conducted in central Uganda on trypanosome species. Samples collected at baseline (before application of treatment), three months and nine months post-intervention were screened for trypanosome infections using species-specific PCR reactions for *T. brucei* s.l., *T. vivax* and *T. congolense* Savannah.

The analyses of the results indicated the success of the mass treatment programme in reducing the prevalence of trypanosome species from animals three months post-intervention. The re-infection of the animal population with trypanosomes in the absence of continued treatment as seen nine months post-intervention indicated continued challenge by infected tsetse flies or possibly movement of infected animals into the study area. The results denote that in the absence of sustained intervention, the trypanosome level returned to pre-treatment levels, indicating that continuous treatment and follow up is necessary to control the infection of the animal host and therefore to reduce the risk of human to get infection.

Mixed infection of the cattle in Uganda showed a low prevalence rate that was within the range of 0.2%-2.1% of the rate obtained with other studies. It was decided to investigate mixed infections in the fly vector. Although most of these infections were *T. brucei* s.l./*T. vivax*, it was decided to use *T. b. brucei* and *T. congolense* as both these trypanosomes inhabit the midgut of the tsetse fly, whereas *T. vivax* completes its lifecycle exclusively within the mouthparts.

8.6 Mixed infection

Field studies have revealed that many vertebrate species which serve as natural hosts of *Glossina* often carry mixed infections of *T. brucei* s.l., *T. congolense* species and/or *T. vivax*. In the current study, during the evaluation of SOS campaign's impact on trypanosome infections in Uganda, the extenet of mixed infection was compared to other field studies in the vertebrate host. The comparison showed that the extent of mixed infection in the vertebrate host ranged from 0.2%-2.1%, with the

extent in the current study 1.7% which is in the same range. In the wild tsetse host, the extent of mixed infection ranged from 1.3%-7.1% in different areas. Although mixed infection is evident in both vertebrate and invertebrate hosts, no studies have looked at the relation between the different trypanosome species in the vertebrate host or vector. Except for few studies that looked at the relation between different species using conventional PCR reactions which give an answer of presence or absence of the infection. Only one study looked at quantifying the number of trypanosomes in mixed infection by labelling the trypanosomes with fluorescence for the quantification. The current study evaluated and optimised qPCR reactions to be used for quantification of trypanosomes in mixed infection of the vertebrate and vector host. The mixed infection of laboratory *Glossina* species and quantification of the infection conducted in Chapter VII suggests the possibility that a form of competition exists between *T. b. brucei* and *T. congolense* Savannah when they co-exist in the fly midgut. However, more research is required to determine the nature of such type of competition between the two species. The work did show that it was possible to accurately quantify the parasitaemia in tsetse flies and further studies could investigate if this technique could be applied to wild flies.

8.7 Recommendations for the future of blood samples from animals in Africa

Successful molecular diagnosis requires the availability of genomic material of an appropriate quality and concentration to be present within the sample under examination. Therefore based on the work presented in this thesis, blood samples should be collected onto FTA®cards where they can be easily transported back to the laboratory for processing. At the laboratory, material from the card should be eluted using Chelex®100 whereby PCR using species-specific primers should take place. Recently, several other ITS primer sets to diagnose trypanosome samples have been published (Adams *et al.*, 2006; Desquesnes, 1997; Geysen *et al.*, 2003; Malele *et al.*, 2003; Maslov *et al.*, 1996; Njiru *et al.*, 2005) and it would be pertinent to compare these primer sets to the ITS primer set developed by Cox *et al*. (2005) and to the species-specific primer sets used in the current work. As development of an ITS based reaction would greatly reduce the cost and work time in processing samples.

8.8 Final thought

This thesis concentrated on molecular diagnosis of African trypanosomes. The significant finding of this thesis was that the FTA protocol using ITS primers described by Cox *et al*. (2005) has limitations for the diagnosis of pathogenic trypanosomes. This was done through the comparison of ITS-PCR to species-specific PCR reactions. As described in Section 8.7 above, a new protocol for the diagnosis of African trypanosomes has been put forward which should improve the diagnosis of this disease in cattle. Accurate determination of the prevalence of this disease is essential to the control and

management of pathogenic trypanosomes particularly where human infective forms are present. The Stamp out Sleeping Sickness campaign has been successful in reducing incidence of trypanosome infection in domestic cattle in Uganda. Although the knock down is only evident for three months it is important that the correct primer set is used to diagnose these infections. If one relied only on the ITS protocol described by Cox *et al*. (2005), then it is possible that many *T. brucei* s.l. infections could be missed. As such an area may be deemed free of this species of trypanosome, therefore control operations may cease too early potentially allowing human infective forms to cross over to human populations. Unchecked this could lead to a new epidemic of Rhodesian sleeping sickness which could place a high burden on a health system already overstretched by other diseases (HIV/AIDS, tuberculosis and malaria).

The other significant outcome of this thesis is that it is possible to measure trypanosome populations in the midgut of the tsetse fly. This could have important implications when looking at infections in wild tsetse flies. As PCR technology has now been applied to wild tsetse populations, it has become apparent that many more flies could be infected than are diagnosed by microscopy. However, conventional PCR can not discriminate between an active infection and trypanosomes that may just of been imbibed with the bloodmeal. Therefore the use of qPCR to measure trypanosome populations in wild tsetse flies may allow the discrimination of active as opposed to non active infections. This protocol could be used also to quantify parasitemia of T. brucei s.l. and T. congolense Savannah and therefore allow monitoring of disease progression in samples collected from animal hosts.

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